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Treball Final de Grau

Formation and characterization of multiple W/W/W emulsions.

Formació i caracterització d'emulsions múltiples W/W/W.

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B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

“The first principles of the universe are atoms and empty space; everything else is merely thought to exist.”

Democritus

En primer lloc, m’agradaria agrair a l’Institut de Química Avançada de Catalunya (IQAC), al Consell Superior d’Investigacions Científiques (CSIC) i, en concret, al Dr. Jordi Esquena Moret, el poder formar part del seu grup de recerca per dur a terme la realització del meu treball final de grau. També donar les gràcies a la Dra. Maria Sarret Pons per preocupar-se pels interessos dels alumnes i obrir-me aquesta porta al món de la investigació. També per haver-me aconsellat i guiat durant tot aquest projecte.

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REPORT

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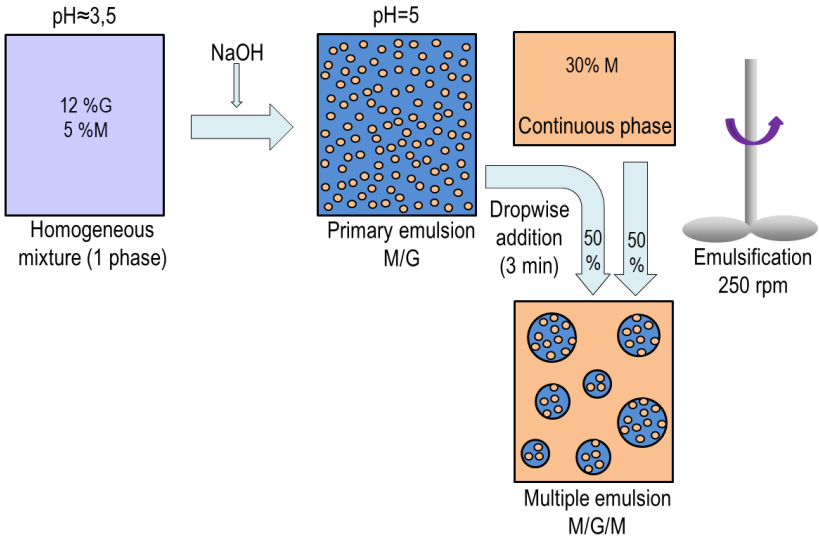
1. SUMMARY

This project was focused in the preparation, stabilization and characterization of multiple water-in-water-in-water W/W/W emulsions. These emulsions are very interesting, because they constitute oil-free dispersions, and very few studies can be found in scientific literature. The studied aqueous two-phase system is composed of gelatin from bovine skin and maltodextrin. These components have been chosen due to their biocompatibility and low cost. The absence of surfactants and nanoparticles makes multiple W/W/W emulsions very biocompatible to be used for pharmaceuticals applications.

In previous work, phase behaviour in maltodextrin-gelatin mixtures have been studied. These colloidal systems can be prepared by mixing two immiscible water-soluble polymers in aqueous solution, such as a polysaccharide and a protein. These systems show complex phase behaviours, which can be controlled by changing pH and ionic strength.

In the present work, the formation of multiple water-based emulsions by two different methods has been studied. The two-step method consists in a first preparation of the primary emulsion followed by a dropwise addition to an external phase. The other method is based on inducing a secondary phase separation in simple emulsions droplets by decreasing the temperature. The emulsions have been characterized using fluorescence microscopy and differential interference contrast microscopy. Moreover, parameters influencing the structure and preparation have been optimized, the optimal conditions are shown in Figure. Stability of dispersions has been improved by gelifiying the gelatin phase, either by cooling down or crosslinking with genipin.



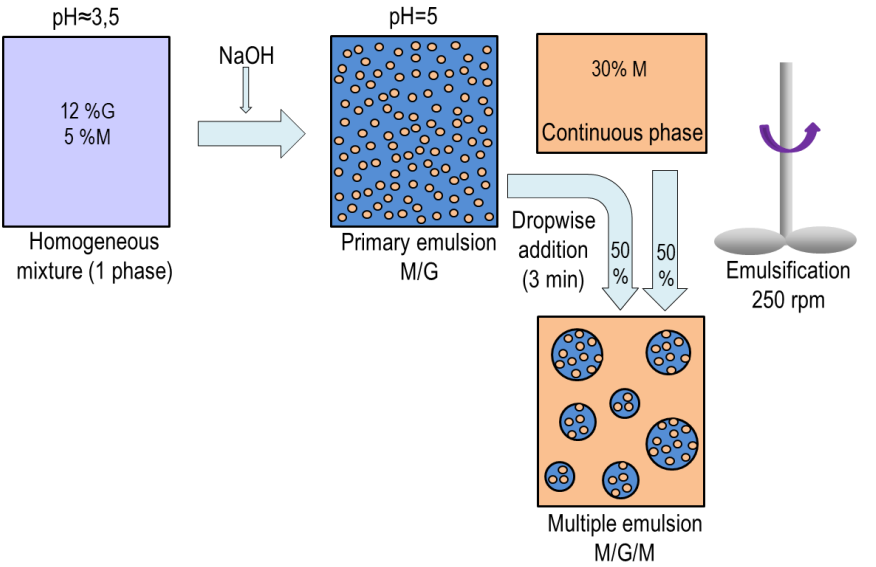
Keywords: W/W/W emulsions, multiple emulsions, crosslinking, colloidal stability, phase behaviour.

2. RESUM

L'objectiu d'aquest treball és la preparació, l'estabilització i la caracterització d'emulsions múltiples aigua-en-aigua-en-aigua (W/W/W). Aquestes emulsions són molt interessants ja que constitueixen dispersions lliures d'oli i gairebé no han estat descrites a la literatura científica. El sistema estudiat es compon de gelatina i maltodextrina , les quals formen un sistema aquós amb dues fases sota certes condicions. Aquests components s'han elegit degut a la seva biocompatibilitat i assequibilitat. L'absència de substàncies externes, com tensioactius o nanopartícules, fan que el sistema sigui biocompatible i pugui ser utilitzat en aplicacions farmacèutiques.

En treballs anteriors es va estudiar el comportament fàsic del sistema gelatina-maltodextrina. Aquest tipus de sistemes col·loidals s'obtenen barrejant dos polímers incompatibles solubles en aigua, normalment polisacàrids i proteïnes. Aquests sistemes tenen un complex comportament fàsic que pot ser controlat amb el pH i la força iònica.

En el treball presentat, s'ha estudiat la formació de emulsions múltiples en sistemes aquosos per dos mètodes diferents. El mètode de dues etapes consisteix en la formació inicial d'una emulsió simple, anomenada primària, que serà posteriorment afegida, gota a gota, a una fase externa. L'altre mètode es basa en la inducció d'una separació de fases secundària a les gotes d'una emulsió simple disminuint la temperatura. Les emulsions obtingudes s'han caracteritzat per microscòpia de fluorescència i per microscòpia diferencial de contrast d'interferència. A més, s'han optimitzat els paràmetres que influeixen en la preparació i l'estructura d'aquests sistemes, els òptims es mostren a la Figura. I finalment, s'ha dut a terme una millora de l'estabilitat per refredament i per entrecreuament amb genipina.



Paraules clau: emulsions W/W/W, emulsions múltiples, entrecreuament, estabilitat col·loidal, comportament fàsic.

3.INTRODUCTION

3.1. COLLOIDAL SYSTEMS

A colloidal dispersion is a heterogeneous system which consist of two or more immiscible phases. One phase is dispersed in the other in form of particles or droplets. A common characteristic of colloids is that at least one dimension is in the 1nm-1μm range [1]. Particles exist in a dispersed state, and practically all substances, whether solids, liquids, or gases can form a colloid.

A colloidal system contains particles of colloidal size spread throughout a dispersing medium. They are usually classified based on their physico-chemical characteristics and the interaction between the dispersed and the continuous phase. A colloidal system has a cloudy appearance because it scatters light.

Application of colloids are unlimited. They play a very significant role in nature and in our daily life. Pharmaceutical products, paints, inks and food formulations are examples. In pharmaceutical applications, a Colloidal Drug Delivery system can be an advancing technology and bring revolutionary changes in pharma and health sciences fields. Despite their finite stability, dispersed colloidal systems have numerous advantages as drug delivery systems. By encapsulating a drug within a colloidal system, the physico-chemical properties of the system determine the biodistribution of the drug, enhancing its therapeutic impact.

The purpose of encapsulation is to protect the drug, to prolong its degradation, to optimize its targeting, to reduce its accumulation in healthy organs, to shrink its potential toxicity, and/or to regulate its release either by natural processes or by external stimuli [2].

3.2. EMULSIONS

Emulsions consist of two immiscible liquids mixtures in which spherical liquid droplets (dispersed phase) are dispersed in a liquid medium (continuous phase). An emulsion is normally denoted by the symbol O/W if the continuous phase is an aqueous solution and by W/O if the

continuous phase is an organic liquid (oil). Emulsions are a very important type of colloids, which are found in the agricultural, pharmaceutical, food and health care industries, for example.

In emulsion formation, the internal phase is subdivided into many small droplets. The change in free energy for emulsion formation has two contributions [3] :

$$\Delta_f \mathrm{G} = \Delta A\gamma - T\Delta S$$

where A is the surface of the drop and γ is the surface tension.

- Surface energy term (positive contribution to total energy)

- Entropy term (negative contribution to total energy)

In most cases, the surface term is higher and total free energy balance is positive, therefore, the formation of emulsions is non-spontaneous, and they are thermodynamically unstable. The emulsion will breakdown by flocculation, coalescence, Ostwald ripening, or combination of all these processes (Figure 3.1) if no stabilization mechanism is used.

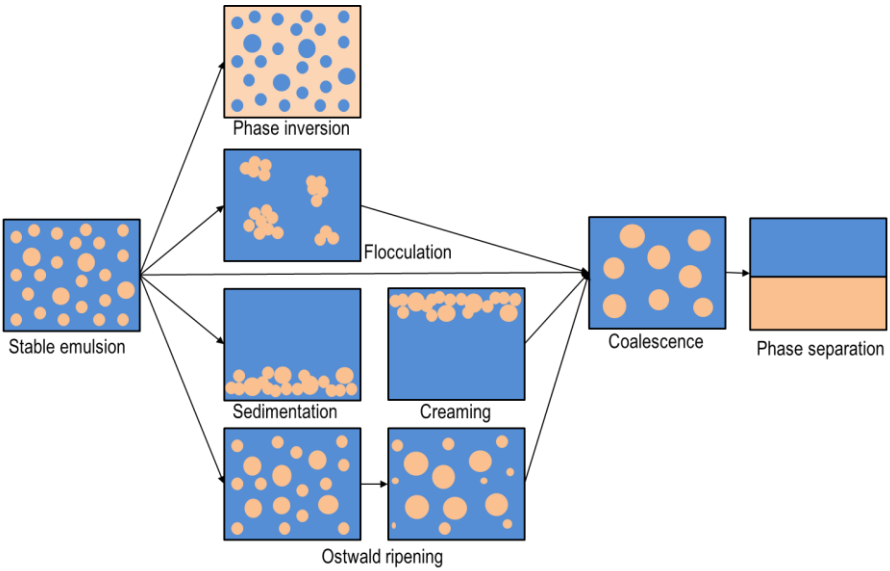


Figure 3.1. Emulsion breakdown mechanisms. Different phases which can be oil or water are represented in different colours.

Generally, surfactant molecules are added, in order to stabilize the dispersion by reducing the interfacial tension. The system becomes kinetically stable in presence of these stabilizers, which reduce surface tension and generate an energy barrier between the droplets.

3.2.1. Multiple emulsions

Multiple emulsions are complex systems where droplets of one liquid are dispersed inside larger droplets of a second liquid (primary emulsion), which are likewise dispersed in the final continuous phase (secondary emulsion). Since the outer phase of one emulsion is the inner one of the other, they are also called 'double emulsions' (Figure 3.2). Multiple emulsions might be the 'oil-in-water-in-oil' (O/W/O) type or the reverse (W/O/W). Their primary application is to slow down the release of hydrophilic drugs.

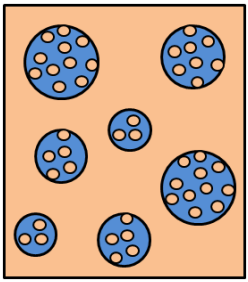


Figure 3.2. Multiple emulsion scheme. Different phases which can be oil or water are represented in different colours.

By encapsulating in the inner emulsions, the active component must pass through the droplets of the outer emulsion to be released. In pharmacology, these emulsions have been used as micro-encapsulation carriers for hormones, anti-cancer agents, etc. They have also been used in cosmetics for easy application of creams with encapsulated compounds [4]**.** Besides that, multiple emulsions of water-based systems are less known. It is interesting to see if it is possible to obtain multiple emulsions of water without the oil.

3.2.2. Water-in-water emulsions

Water-in-oil (W/O) or oil-in-water (O/W) emulsions have since long time been used as templates for nano- and microparticles. Using water-in-water (W/W) emulsions for drug delivery is an interesting alternative for oil-containing emulsions [4]. The surfactants required for their stabilization can be a disadvantage for drug delivery where good biocompatibility is required.

Water-in-water (W/W) emulsions are formed by mixing two incompatible water-soluble macromolecules, leading to two phases formation. The dispersed phase will be enriched with one macromolecule and the continuous phase enriched with the other. The interfacial tension between the two phases is orders of magnitude smaller, of order $\mu N\ m^{-1}$ as opposed to $mN\ m^{-1}$

[5] for oil/water interfaces [6]. W/W emulsions cannot be stabilized by surfactants because the polarity of the two phases is very similar. Functionalizing water-in-water emulsions is the aim for the development of cosmetics, pharmaceuticals or food industry based on oil-free emulsions [4].

The different situations in phase behaviour depend on the repulsion or attraction between the two different hydrophilic polymers when mixing them [7] (Figure 3.3).

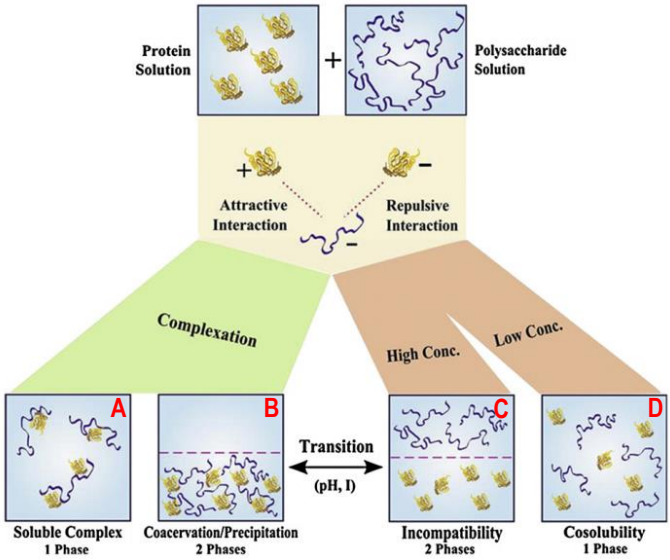


Figure 3.3. Scheme showing the four different phase situations of proteins and polysaccharides either attraction or repulsion between macromolecules [8].

Figure 3.3 C represents a segregative phase separation induced by repulsive interactions between the polymers at high macromolecule concentrations. This leads to the formation of Aqueous Two-Phase Systems (ATPS) which will be the focus in the present work. Two aqueous solutions in thermodynamic equilibrium constitute this ATPS. In them, each phase is enriched with one of the polymers and saturated with the other polymer [9]. Emulsification of such two-phase systems leads to the formation of water-in-water emulsions.

When the molecules have no opposite charges, the repulsive interaction between the molecules might lead to thermodynamic incompatibility, and eventually phase separation. The phase behaviour can be adjusted by changing pH and ionic strength conditions [7].

3.2.2.1. Applications of W/W emulsions

Water-in-water emulsions have been known for a long time [10]. They were discovered by serendipity in 1896 by Beijerinck, when he observed droplets when mixing starch solutions with gelatin [7]. In the recent years, studies of these systems have led to essential discoveries and to new technological features.

For a long time, ATPS have been used for the extraction of biomolecules avoiding the use of hydrophobic phases to which molecules may be sensitive. The use of an oil phase could be problematic in fat-free food products and in other emulsion applications, in which replacement with another aqueous phase would be more desirable. Their environmentally friendly, biocompatible or even food-grade qualities make these systems suitable to several applications. In fact, many available food products are W/W emulsions that consists of mixtures of protein and polysaccharides, in spite of the fact that often the presence of W/W emulsions in these products it is not completely understood [7].

The micro- and nanostructure of aqueous formulations of polymers are important aspects of the functionality of these formulations. Examples are water-based paints, encapsulation of food ingredients and pharmaceuticals, and the texture customers feel when eating certain food [11].

Many biopolymers, such as polysaccharides and proteins mentioned before, can be used to create a large amount of delivery systems suitable for encapsulating, protecting, and controlled delivery of active components [12]. Simple processing operations, such as mixing and stirring, can be used to create these systems.

3.3. MALTODEXTRIN

Maltodextrin has been widely used in the food industry, especially as a fat replacement. It is an oligosaccharide produced from the acid hydrolysis and/or enzymatic treatment of corn starch. It is generally composed of glucose, maltose, maltotriose, and glucose polymers (Figure 3.6). Its final concentration varies depending on the method and degree of hydrolysis and it is chemically expressed as a “dextrose equivalent” (DE). A high DE is indicative of an high amount of reducing sugars, which results in the rapid metabolism of the product [13]. From the DE value, physical properties such as glass transition temperature, hygroscopicity, and colligative properties can be estimated [14]. Maltodextrin used in the present work is dextrose equivalent 4.0-7.0.

Among the known drying agents available, maltodextrin is the most used in food industry because its competitive price, and adaptability.

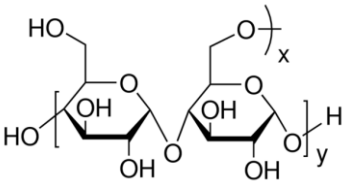


Figure 3.6. Maltodextrin [15].

3.4. GELATIN

Gelatin is a heterogeneous combination of water-soluble proteins of high average molecular masses resulting from collagen. Proteins are extracted by boiling the relevant skin, tendons, ligaments, bones, etc. in water. Type A gelatin is derived from acid-cured tissue. Type B is derived from lime-cured tissue [16]. The amino acid composition and sequence in gelatin depends on the source [17]. The product used in this work is derived from bovine skin (Type B).

Collagen is a very important protein in the animal kingdom and consists of three helical polypeptide chains wound around each other and connected by intermolecular cross-links. Gelatin is considered a hydrocolloid and has been widely used in food applications. Its biocompatibility makes gelatin a suitable delivery vehicle for release of active biomolecules. Gelatin solutions in the concentration range of approximately 1-50 wt% can form thermoreversible gels [9]. This thermal behaviour results in high versatility in gelatin applications. The formed gels are polymer networks which expands throughout its whole volume by a fluid, usually water [18]. Regarding its structure, gelatin molecules consist mainly of repeating sequences of glycine-proline-hydroxyproline triplets. Structure basis is built by these gelling blocks. Ordered rigid triple-helix segments compose the network which resembles the nature of collagen state. Hydrogen bonds and other intermolecular cross-links serve as stabilization of the helix structure [19]. At temperatures above its gelation point, gelatin monomers are independently distributed in the solution. Cooling down promotes hydrogen bonds formation and the monomers aggregate into oligomers, therefore a random coil conformation is formed in the liquid. When going to lower temperatures, these coils are ordered in a simple helix conformation. When eventually the polymer gelifies, a crossover from single to triple helix occurs (Figure 3.4) [9].

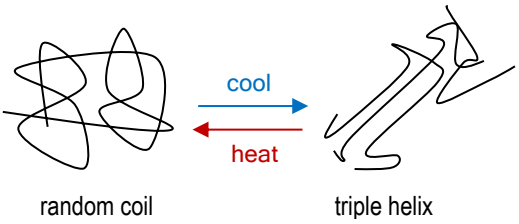


Figure 3.4. Transition from coil to helix of gelatin induced by temperature changes [9].

The Bloom index is an important physicochemical parameter which represents the amount of triple-helix in gelatin. It is measured by gel strength and determines the mechanical, rheological, and thermal characteristics as well as biodegradation behaviours and the release mechanisms [20].

A gel's three-dimensional structure and its stability is determined by physical or chemical crosslinks. Physical crosslinking are made up of hydrogen bonds, ionic interactions, and hydrophobic forces. Gels can also be chemically crosslinked using crosslinker molecules such as genipin [19]. Chemical crosslinking uses the functional groups present in the amino acids as reaction points.

3.5. GENIPIN

Cross-linking is one of the most important methods to modify polymer networks. By using a crosslinker, the polymer adopts a fixed conformation that prevents it from being disrupted by external conditions. Many properties of microgels and microparticles can be defined by the crosslinker nature and its structure [21].

Genipin is a natural crosslinker that comes from geniposide, extracted from gardenia seeds, and has low cytotoxicity [22]. It is obtained via enzymatic hydrolysis with β-glucosidase. Genipin reacts chemically with free amine groups of the proteins, and its reaction time is highly variable affected by pH and both protein and genipin concentrations. The major disadvantage of genipin is that crosslinking reaction results in a dark blue colour in presence of air. This is caused by oxygen radical-induced polymerization of genipin and dehydrogenation of intermediate compounds, following the ring-opening reaction described in Figure 3.5 A. [23].

Two reaction mechanism of genipin with primary amine groups of gelatin have been suggested (Figure 3.5) [23]. Other biopolymers containing these reacting groups, mainly from lysine or arginine residues, go thought the same mechanisms.

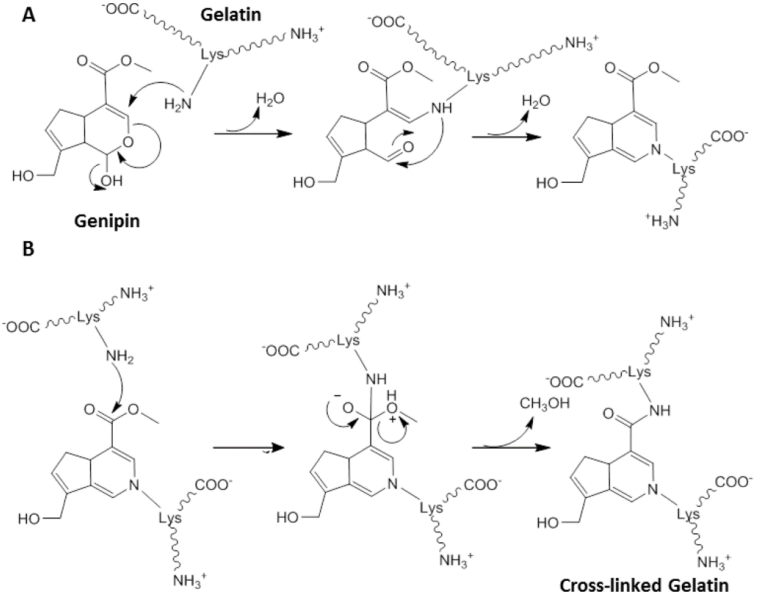


Figure 3.5. Crosslinking mechanism of genipin includes two reactions: (A) Nucleophilic attack of primary amino group of gelatin to genipin ring. (B) SN_2 nucleophilic substitution between genipin ester group and another primary amine group. Figure reproduced from [9].

In W/W emulsions, if gelatin forms the dispersed phase in the emulsion, it can gellify and trap the system in a metastable state. This represents a great advantage of using gelatin/maltodextrin emulsions as templates for microgel preparation. Cooling down and/or crosslinking amino groups, which are solely present in gelatin, allows gelation of gelatin. Multiple maltodextrin-in-gelatin-in-maltodextrin (**M/G/M**) emulsions may be used to encapsulate active components introducing this component in the inner phase and stabilizing the system by gelation. The kinetics release of the active component and the biocompatibility of the system make them suitable for pharmaceutical applications.

4. OBJECTIVES

The current work will focus on the preparation and characterization of simple water-in-water (W/W) emulsions and multiple water-in-water-in-water (W/W/W) emulsions, formulated in a mixture of a protein (gelatin from bovine skin) and a polysaccharide (maltodextin). The main objectives are as follows:

- Evaluation of phase behaviour, in order to determine the regions in which water-in-water-in-water emulsions can be prepared.
- Formation and optimization of multiple W/W/W emulsions.
- Stabilization of W/W/W emulsions by gelation and crosslinking.

The importance of using biocompatible components is highlighted, for possible uses in pharmaceutical and food applications.

5. EXPERIMENTAL SECTION

5.1. MATERIALS

- Gelatin: from bovine skin, 225g Bloom, Type B. Supplied by Sigma Aldrich.

- Maltodextrin: dextrose equivalent 4.0-7.0. Supplied by Sigma Aldrich.

- Sodium hydroxide: NaOH. Pellets, >97%. 40 g/mol. Supplied by Carlo Ebra.

- Genipin used as a crosslinker: $C_{11}O_5H_{14}$, 98% up by HPLC method, with a melting point 120-121ºC and a molecular weight of 226.23 g/mol. Supplied by Challenge Bioproducts CO., LTD.

- Dimethyl sulfoxide (DMSO): $(CH_3)_2SO$ ACS reagent, ≥99.9%, with a molecular weight of 78.13 g/mol. Supplied by Merk-KGaA.

- Fluorescein 5(6)-isothiocyanate: $C_{21}H_{11}NO_5S$ BioReagent, suitable for fluorescence, mixture of 2 components, ≥90% (HPLC). With a molecular weight of 389.38 g/mol. Supplied by Sigma-Aldrich. (**FITC**)

- Rhodamine B isothiocyanate: $C_{29}H_{30}ClN_3O_3S$ BioReagent, suitable for protein labelling. With a molecular weight of 536.08 g/mol. Supplied by Sigma-Aldrich. (**RBITC**)

- Filtered deionized water: H_2O, Milli-Q® water (ultra-pure Millipore water system, Milli-Qplus 185 filter). Supplied by Merck Millipore.

- Acetic acid glacial: CH_3COOH (USP, BP, Ph. Eur.) pure, pharma grade. 60.05g/mol. Supplied by Panreac ITW Companies

- Ethanol. Bulk ethanol. 46.07 g/mol. 95%. Supplied by Alcoholes Montplet.

- Pyridine anhydrous, 99,8%. C_5H_5N. With a molecular weight of 79.10 g/mol. Supplied by Sigma-Aldrich.

- Dibutyltin dilaureate: $C_{32}H_{64}O_4Sn$. With a molecular weight of 631.56 g/mol. Supplied by Sigma-Aldrich.

5.2. EQUIPMENT

- Analytical balance Mettler Toledo AB204-S/FACT. Maximum: 220g
- Basics magnetic stirrer hot plate Heidolph MR Hei-Standard with temperature controlled by means of Heidolph EKT3001 probe (maximum stirring capacity: 1500 rpm).
- Basics magnetic stirrer hot plate IKA® RCT. (maximum stirring capacity: 1500 rpm).
- pH-meter Mettler Toledo Seven Easy.
- Oven KOTTERMANN 2712 (maximum temperature: 250 ºC).
- Optical microscope Olympus BX51TRF-6
- ULTRA-TURRAX T25. Stick s25n-10g
- Vacuum pump: VACCUBRAND MZ2CNT

5.3. METHODS

5.3.1. Fluorescent labelling of molecules

Maltodextrin was covalently labelled with FITC or RBITC by the method used before in the research group [4]. Labelling the inner phase of primary emulsions will provide additional contrast between the phases, this will allow to correctly characterize the emulsions. A solution of 10 grams of maltodextrin and 25 mg of isothiocyanate in 100 ml of DMSO was prepared adding a few drops of pyridine and 200 mg dibutylindilaureate. The solution was incubated at 65°C for 2 h under continuous stirring. After 2 h the maltodextrin was precipitated with ethanol (95% v/v), filtered and washed several times with ethanol. The labelled solid was dried in the oven at 45°C for 5 h.

In some cases, labelling gelatin was needed either with FITC or RBITC. A solution of gelatin was prepared adding 1µl of isothiocyanate solution 2 wt% in DMSO for each ml of gelatin solution and stirred for homogenization.

5.3.2. Preparation of primary emulsions

The emulsions were prepared either by a high energy method (Ultraturrax at 8000 rpm) or by phase transition. Solutions of Gelatin (25 wt%) and Maltodextrin (30 wt%) were prepared as follows: maltodextrin powder was stirred with Milli-Q water at 95°C for approximately 20-30 minutes. Gelatin solutions were prepared likewise but were heated at 50°C to prevent hydrolysis of gelatin chains at high temperatures.

5.3.2.1. Preparation of primary emulsions by phase transition

The emulsions formed by the phase transition method were prepared by adding water, acetic acid, gelatin solution and finally maltodextrin solution, in this order. The solution was then mixed with a magnetic stirrer for 5 min at 500 rpm an at 50°C for 8 g of emulsion in a water bath. The final concentration of acetic acid was 3.3%. Then 0.12 g sodium hydroxide was added for neutralization, changing the pH value from ≈ 3.5 to 5.

5.3.2.2 Preparation of primary emulsions by high energy method

In order to form the emulsions with the Ultraturrax, 16 grams were prepared adding the components in the following order: first water, then gelatin and lastly maltodextrin, both in solution. The mixture was then stirred for 2 mins at 8000 rpm in a water bath at a temperature of 50°C.

5.3.3. Preparation of multiple emulsions

5.3.3.1. Preparation of multiple emulsions by two-step method

Multiple emulsions were formed by a two-step method. The first step was to prepare a primary emulsion of maltodextrin in gelatin by one of the methods described above. This primary emulsion was added dropwise to a maltodextrin phase with different concentrations. In the emulsification process of multiple emulsions, different parameters were considered and their influence in the final properties were studied: primary emulsion/ dispersed phase ratio, the addition of the components, addition time and stirring speed.

5.3.3.2. Preparation of multiple emulsions by temperature change

Compositions close to the binodal line of the phase diagram formed multiple emulsions by cooling the sample. Gelatin and maltodextrin were mixed in a water bath at 50°C forming simple G/M emulsions. Lowering temperature, smaller droplets of maltodextrin were formed in the already gelatin formed droplets.

5.3.4 Stabilization of multiple emulsions

Once the multiple emulsions were obtained, they were stabilized by temperature change or with a crosslinker in different conditions of stirring.

5.3.4.1. Stabilization by temperature change

As the emulsions were prepared at 50°C, the emulsions were cooled in an ice bath or water bath in order to study their stability at lower temperature, to determine if emulsions were stable at body temperature for biological applications. The influence of changing the following cooling conditions was evaluated: water bath temperature, stirring conditions and cooling time.

5.3.4.2. Stabilization using a crosslinker

Genipin was used as crosslinker between gelatin molecules. After several preliminary tests with gelatin solutions, genipin was added at different concentrations between 0.1 and 0.8 wt% in different steps of multiple emulsions preparation. In primary emulsion preparation, it was added in two possible ways: before or after the phase transition. Moreover, experiments were performed by adding genipin in the external phase of multiple emulsions.

5.4. CHARACTERIZATION

5.4.1. Optical Microscopy

With the aim of characterize the obtained emulsions, an Olympus model BX51TRF-6 equipped with a digital camera and controlled with image/video capture software Stream Essential of Olympus was used. The addition of FITC and RBITC before the observation allows to highlight the component of interest from the rest of the sample, being properly studied. For this reason, one of the phases was labelled with FITC or RITC before observing the samples under the microscope. A different filter was used to observe the fluorescein or rhodamine labelled samples.

5.4.1.1 Fluorescence microscopy

The sample is excited with light of the wavelength that is absorbed by the fluorophores, causing them to emit light of longer wavelengths (a different colour than the absorbed light). A spectral emission filter separates the illumination light from the emitted fluorescence. The fluorescing labelled areas shine against a dark background with enough contrast to permit detection.

Fluorochrome is a fluorescent chemical compound which can absorb and then re-radiate light, it is often highly specific and have significant yield in absorption-emission ratios. This makes them extremely valuable in biological applications [24]. In the present work, two fluorochromes are used:

Fluorescein isothiocyanate (FITC) is the original fluorescein molecule functionalized with an isothiocyanate reactive group (-N=C=S), replacing a hydrogen atom on a ring of the fluorescein structure (Figure 5.1 A). FITC is orange in colour with an absorption maximum at 495nm. While excitation, it emits a yellow-green colour with an emission maximum at 525nm. The isothiocyanate group reacts with amino terminal and primary amines in proteins. It has been widely used for the labelling of proteins including antibodies and lecitines [25].

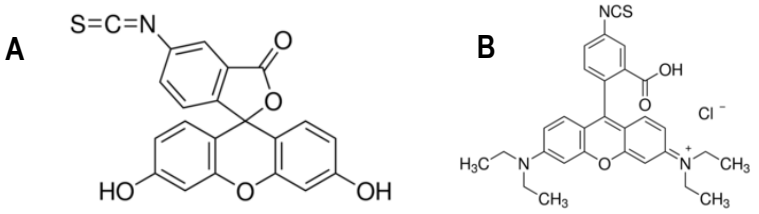


Figure 5.1. A) Fluorescein isothiocyanate molecule [25]. B) Rhodamine B isothiocyanate molecule [26].

Rhodamine B isothiocyanate (Figure 5.1 B) is another organic fluorescence dye and is also widely used as a fluorescent label in a variety of biological applications. It has the same labelling reaction between isothiocyanate and amino groups. It absorbs at 540nm and emits at 573nm, in red colour [26].

5.4.1.2 Differential interference contrast microscopy

Differential interference contrast (DIC) microscopy allows to visualize transparent structures by taking advantage of changes in refractive index [27]. This technique makes objects more visible under the microscopy by using gradients in the optical path length and phase shifts. In this way, it is possible to observe living cells and organisms with the required contrast and resolution. In DIC microscopy, only polarized light is used to illuminate the sample. It uses two rays of polarized light, the combined images appear as if the sample had shadows by the side. It was designed to observe reliefs of very difficult to handle specimens and the ones that cannot be labelled by fluorescence [28].

6. OPTIMIZATION OF PRIMARY EMULSIONS FORMATION

The phase diagram was previously described of the ATPS of gelatin (**G**) and maltodextrin (**M**). (Figure 6.1) [9]. Three regions were observed in a solution at pH 5 (Figure 6.1 A). At lower concentrations, there was one liquid phase (L), and as the concentration increased different phases could be observed. Compositions with more than 5 wt% of each polymer lead to a three-phase region of two liquids and a precipitate(L_1+L_2+S). A middle region (L+S) with the presence of one liquid and a precipitate was also observed.

At a pH ≈ 3.5 the polymers were soluble at low concentrations (Figure 6.1 B). However, the mixture separated into two phases at higher concentrations, a liquid and a precipitate (L+S).

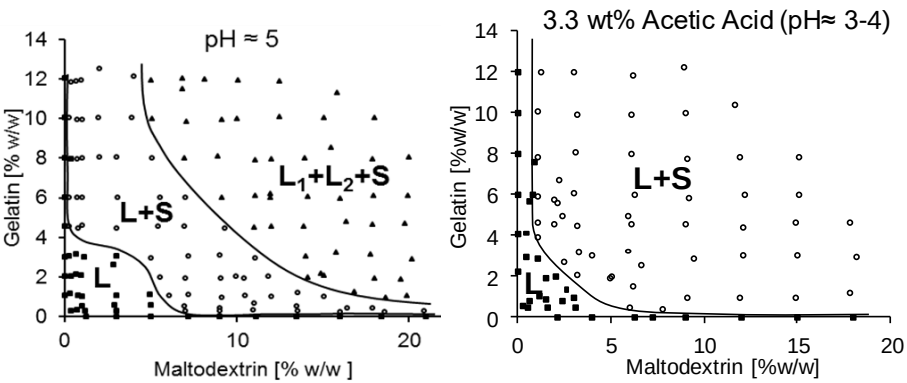


Figure 6.1. Phase behaviour of gelatin and maltodextrin mixtures after 5 days at 50°C [9].

All the mixtures were prepared at low pH given by the acetic acid added in the solution (3.3 wt%). In these conditions, all the emulsions studied by phase transition method (Table 6.1) were in the region in which only one liquid phase exists.

The isoelectric point (**pI**) denotes the pH value when a macromolecule has no overall electrical charge. In solutions below its pI, gelatin acid groups are protonated and uncharged. Therefore,

repulsion between gelatin and maltodextrin decreases in acidic pH. In this case, polymers are dispersed throughout the system more uniformly, and form a single phase [29]. The addition of sodium hydroxide increases the pH of the mixture, and thus introduces negative charges on gelatin molecules, due to deprotonation of carboxylic groups. These negative charges could lead to phase separation because the interaction of gelatin and maltodextrin becomes repulsive. The compositions studied are in the region of two liquid phases at final pH.

Table 6.1. Composition of biopolymers in primary emulsion. PT: Phase transition. HEM: High energy method.

Sample	Gelatin (wt%)	Maltodextrin (wt%)	Method	Observations
1A	12	5	PT	droplets 3-5μm
1B	5	12	PT	spinodal decomposition
1C	4	20	PT	droplets 12-30μm
1D	12	5	HEM	droplets 8-15μm

Compositions A and C will lead to emulsion formation (Figure 6.1 A and C), the first one maltodextrin-in-gelatin because gelatin is more present in the mixture. The mixture C, which contains less gelatin, will produce gelatin droplets in a continuous phase enriched with maltodextrin. Maltodextrin droplets were different in size than gelatin ones, due to the higher viscosity of gelatin, that produces bigger droplets.

The emulsion droplets contain mainly one polymer, but they are also saturated with the other polymer, of which contain a small concentration. If the tie line of a phase diagram is known, the composition of the equilibrium phases can be determined. It corresponds to the end points of the tie line on the binodal line. This line separates the regions with different phases. However, tie lines of the M/G system have not been studied.

The image observed in Figure 6.2 of sample 1B shows the typical bicontinuous pattern of spinodal decomposition. This mechanism differs from the classical nucleation and growth (the mechanism in compositions A and C). Phase separation by nucleation and growth starts by the formation of small particles which increase in size as function of time. This mechanism leads to a polydisperse systems of growing droplets that eventually phase separate [30]. The phase separation due to spinodal decomposition is much more defined and occurs uniformly throughout

the sample, instead of nucleation in discrete points. Simultaneous nucleation of the two phases at very similar rates causes an unstable situation of bicontinuous emulsion, where eventually the two phases separate.

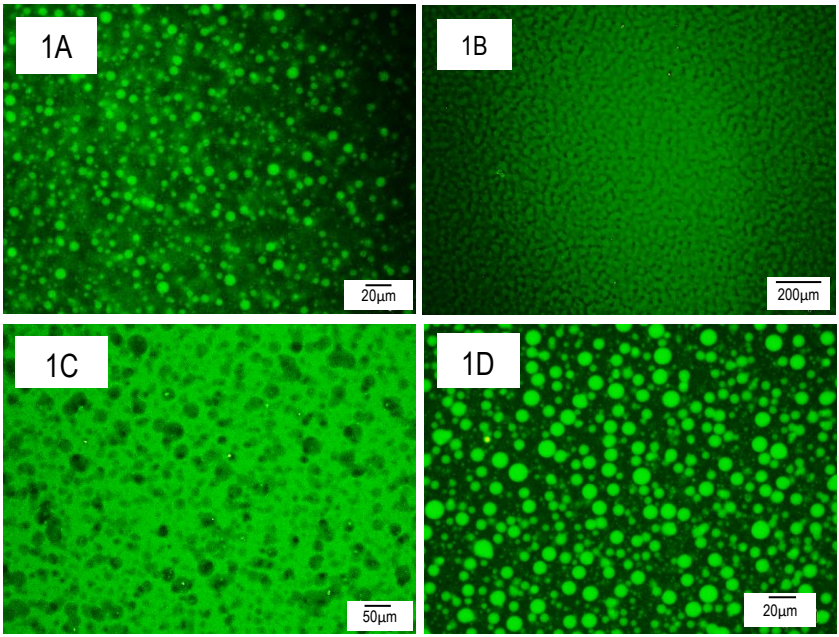


Figure 6.2. Phase separation in compositions given in Table 6.1. In all cases, maltodextrin was labelled with FITC and the images were taken just after the preparation of the sample.

The emulsion obtained by Ultraturrax (Sample 1D) had bigger droplets compared to that with the same composition obtained by phase transition. However, it was easier to prepare. Since the formation of a W/W emulsion with a composition of 12% G and 5% M by phase transition method formed the smallest droplet size, this composition was chosen to be further continued with for the formation of multiple emulsions.

Gelation of the obtained primary emulsion by cooling it at room temperature, led to gel formation, due to triple helix conformation, and breakdown of maltodextrin droplets. Fluorescence was seen throughout all the gelatin network. The sample was observed again after re-heating, from room temperature to 50°C. Then, maltodextrin droplets were slightly bigger than the initial emulsion. Nevertheless, it was considered that maltodextrin-in-gelatin emulsions were stable enough and the formation of multiple emulsions could be possible.

7. MULTIPLE EMULSIONS FORMATION BY TWO-STEP METHOD

The primary emulsion (**PE**) was added dropwise to a maltodextrin phase, initially for 3 minutes under agitation with a magnetic stirrer. Each time, the primary emulsion was prepared just before using it to form multiple emulsions (**ME**) and all of them with the composition studied before, 12% G and 5% M by phase transition method. The effect of stirring conditions while adding PE was firstly studied (Figure 7.1).

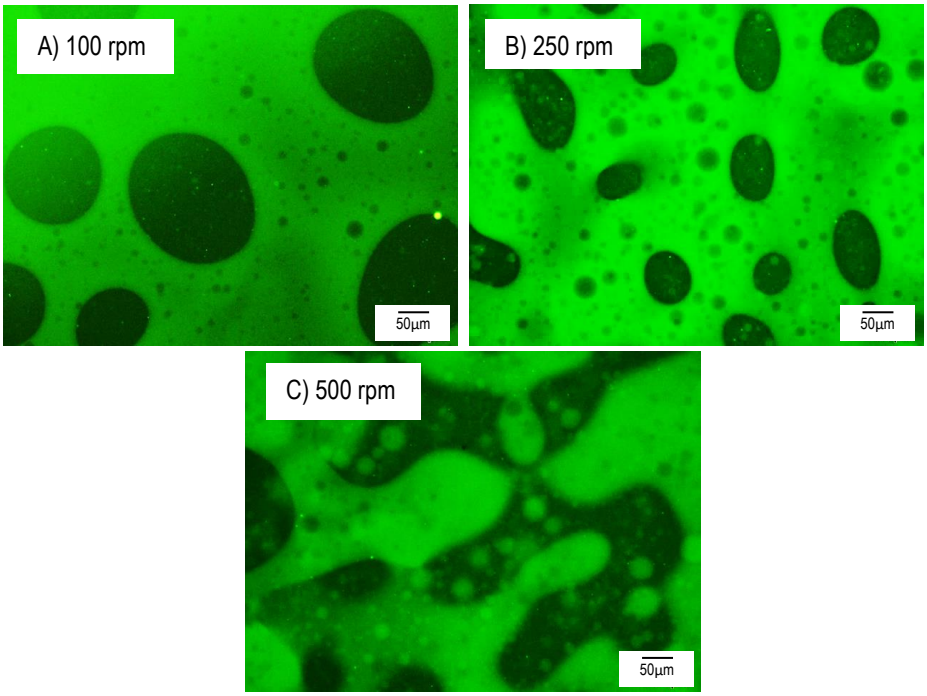


Figure 7.1. Multiple emulsions prepared at different stirring conditions. All samples were composed by 40% of M phase (30% M) and 60% of primary emulsion. In all cases, maltodextrin was labelled with FITC, and the images were taken just after the preparation of the sample.

All stirring conditions lead to the presence of simple G/M emulsion droplets. Slower agitation produces big multiple emulsions droplets (diameter ≈ 200μm) with small inner droplets of maltodextrin. However, sample is not homogenous as some parts remain as simple emulsions M/G. Agitation at 250 rpm leads to multiple emulsion droplets about 40μm and their inner droplets are bigger than the ones mentioned before. Faster agitation breaks primary emulsion droplets and multiple emulsions are not clearly formed. The best result was achieved at 250 rpm, and thus, this agitation speed was used for further experiments.

Time of addition has also been studied. One drop every two seconds formed emulsions as the ones seen before (Figure 6.1 B). Faster addition speed leads to less ME droplets and more simple G/M droplets. Slower addition affects the stability as the emulsion breaks as time elapses. Different ratios tested between primary emulsion and continuous phase (**CP**) are shown in Table 7.1.

Table 7.1. Multiple emulsions prepared at different Primary emulsion/Continuous ratios.

Sample	PE (wt%)	CP (wt%)	Observations
2A	60	40	Presence of two fractions: M/G and G/M emulsions.
2B	50	50	M/G/M multiple emulsions droplets, coexisting with G/M droplets.
2C	40	60	
2D	35	65	Simple emulsion G/M type.

Using high concentration of primary emulsions (Sample 2A) not all the droplets are emulsified. The multiple M/G/M emulsions tend to be very unstable (Sample 2B and 2C). On the other side, low ratios of primary emulsion lead to simple emulsion formation gelatin-in-maltodextrin (sample 2D). Although the presence of some small simple gelatin droplets, the most well defined multiple emulsion is achieved with a 50/50 ratio (Sample 2B).

Diffusion across the intermediate gelatin phase can occur if the concentrations in the different phases are not in equilibrium. This could induce a breakdown of multiple emulsions droplets. For this reason, different concentrations of the outer maltodextrin phase were studied. The compositions in Table 7.2 were tested and only the last one formed multiple emulsions (Sample 2H). At compositions between 10 and 30 % spinodal decomposition was observed. At low maltodextrin concentrations (Sample 2E), the final mixture has a composition which corresponds to a single liquid phase (Phase diagram in Figure 6.1). Thus, emulsions are not formed.

Table 7.2. Multiple emulsions prepared with different concentration of maltodextrin in the CP.

Sample	M in CP (wt%)	Final G concentration (wt%)	Final M concentration (wt%)	Observations
2E	10	4.8	8	One single homogenous phase.
2F	15		11	Phase separation by spinodal decomposition.
2G	20		14	
2H	30		20	Formation of multiple emulsions.

Samples 2F and 2G had final compositions, corresponding to a two-liquid region, very close to the binodal line. Compositions in both phases are similar and hence they had comparable nucleation speeds. This led to a mixture of the components while PE addition and a later phase separation by spinodal decomposition.

The systematic study of the ATPS composed of gelatin and maltodextrin concludes that the best conditions to prepare multiple emulsions by two-step method are the ones shown in Figure 7.2.

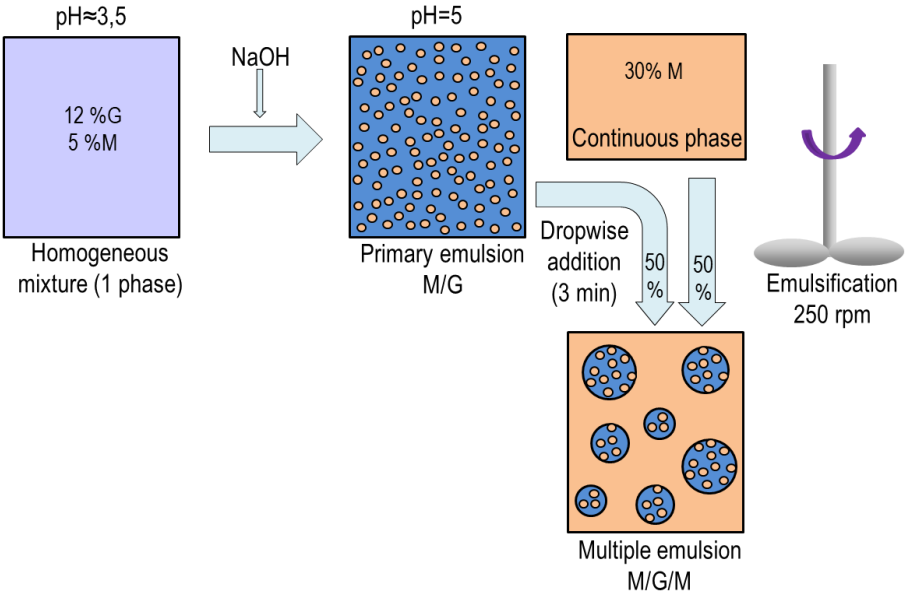


Figure 7.2. Two-steps method scheme with the optimal conditions. Maltodextrin is represented in flesh colour and Gelatine with blue.

The amount of sample used in the preparation of the emulsions was always 8 g. Different volumes would probably need other stirring conditions.

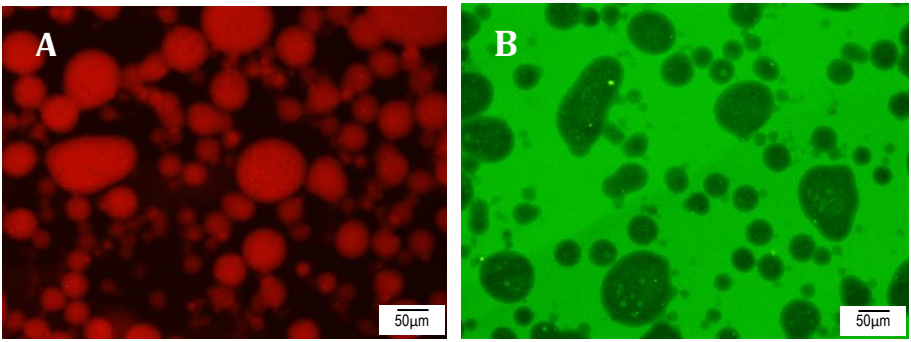


Figure 7.3. Multiple M/G/M emulsions formed with the conditions described above. Images obtained using the Hot stage microscope accessory at 50°C. A) Gelatin phase is labelled with RBITC. B) Maltodextrin phases are labelled with FITC.

Multiple emulsion droplets, 40μm on average, with primary maltodextrin droplets, around 5μm, were obtained using this method (Figure 7.3). The final ME composition was 6% Gelatin and 20% Maltodextrin.

7.1 DIFFUSION OF COMPONENTS

The studied system turned out to be more complex than expected. With the aim to better understand the ME formation, a diffusion study was performed. Only the outer maltodextrin phase was labelled with FITC, and primary emulsion maltodextrin droplets where made up of non-labelled M. The multiple emulsion was observed in the microscope just after its preparation. Inner maltodextrin droplets became also green-coloured (Figure 7.4), which means that there was diffusion from external media to the inner primary droplets.

The inner primary droplets appeared green-coloured very soon, just after the preparation of ME. Therefore, it seems that diffusion of maltodextrin, across the intermediate gelatin phase, is very fast. These results seem reasonable, since there is not any barrier which could slow down diffusion. Water is the same solvent in all regions, and probably, the polymers can freely diffuse from one region to another. It has to be remarked that the interfaces might not have any adsorbed layer that could reduce diffusion. Some experiments were done in order to better characterize this

process. However, technical problems explained in appendix 1 did not allow the diffusion to be described.

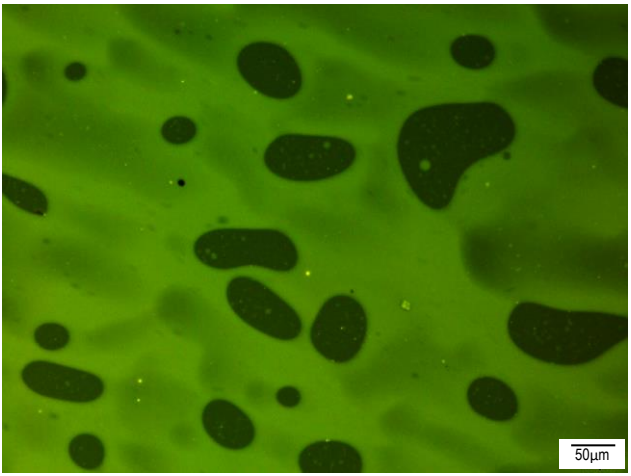


Figure 7.4. Multiple emulsions, with composition of 6% G and 20% M, formed with only CP labelled maltodextrin.

8. STABILITY OF MULTIPLE EMULSIONS

In order to encapsulate a potential active component, multiple emulsion stability was studied. Phase separation occurs at temperatures above gelatin gelation point. Taking emulsions to lower temperatures may lead to gelation of gelatin phase and avoid some emulsion breaking process. For this reason, their stability was studied at different constant temperatures, above and below gelatin gelation point, using a water bath. Faster gelation at 0°C was tested in an ice bath.

8.1. STABILITY AT CONSTANT TEMPERATURE

Multiple emulsions were prepared in water bath at 50°C because gelatin remains liquid and it is easy to manipulate. Keeping the multiple emulsions without any agitation leads to phase separation in approximately 10 minutes. Gelatin forms the upper phase as it is less dense than maltodextrin. When phases are separated, and the sample is kept at room temperature, complete gelation of the upper part is eventually observed.

As the release of active components takes place at body temperature, stability was studied in a water bath at 37°C. After its preparation, multiple emulsion was left in a bath at this temperature. If slow agitation is applied emulsion breakdown is observed as coalescence and creaming also occurs. Non-stirring conditions also lead to phase separation.

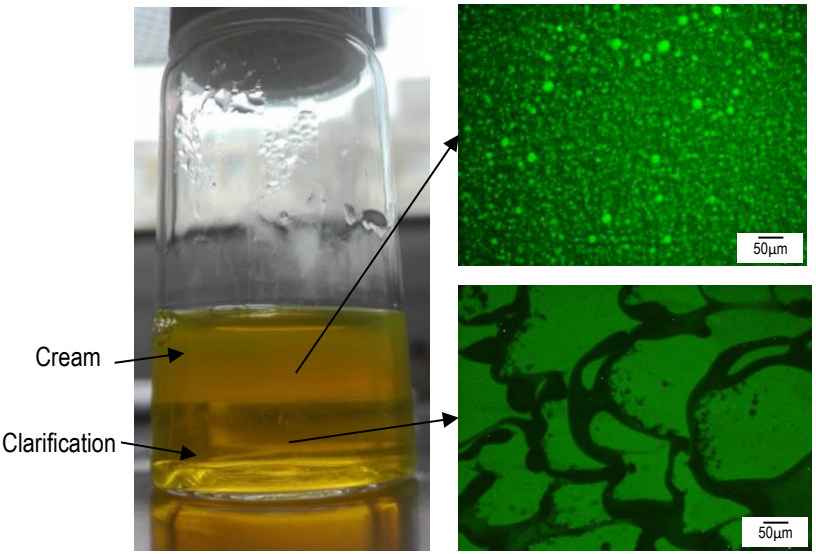


Figure 8.1. Phase separation of multiple emulsion after 3h in a water bath at 37°C. Maltodextrin phase is labelled with FITC.

After creaming, each fraction was observed in the microscope (Figure 8.1). The upper fraction was composed of primary emulsion M/G, and the lower contained mainly maltodextrin. Breakdown mechanism was probably coalescence and creaming as the creamed part, located at the top of the sample, seems to be the primary M/G emulsion.

Stability was also studied at 25°C. This temperature leads to gelation of gelatin. Sample was kept at constant temperature and it was observed in the microscope along time (Figure 8.2). Low stirring conditions were used the first minutes.

Microscopy observations revealed that the multiple emulsion, at 25°C, remained stable only for short periods of time (≈ 12 minutes). As gelation occurs, viscosity increases and coalescence forms gelatin aggregates. Gelation of gelatin phase breaks maltodextrin droplets. After 30 minutes, at 25°C, the sample is completely gelled.

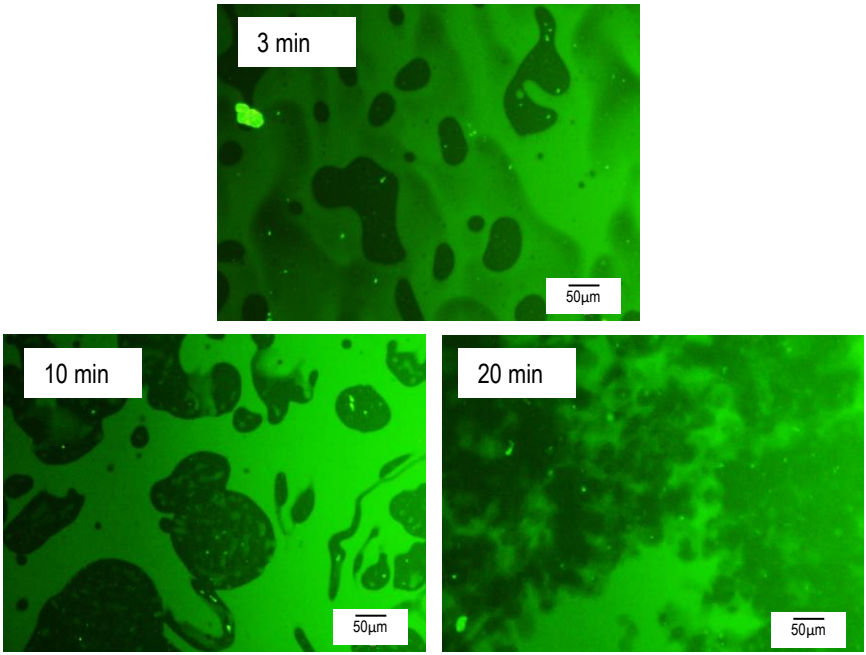


Figure 8.2. Images of a multiple emulsion, taken at different times in a water bath at 25°C. Maltodextrin phases are labelled with FITC.

8.2 GELATION BY DECREASING TEMPERATURE.

Faster gelation was tested using ice baths to cool the sample. The cooling conditions are shown in Table 8.1.

Table 8.1. Different cooling conditions applied after ME formation.

Sample	Cooling time (s)	Stirring (rpm)	Observations
2I	60	500	completed gelation of the sample with emulsion breakdown
2J	60	250	
2K	60	-	
2L	30	manual	
2M	30	250	aggregate formation with emulsion breakdown
2N	10	manual	

None of the conditions described in Table 8.1, lead to multiple emulsion droplets stability. Long cooling times with strong stirring conditions promote coalescence gelling all samples. Shorter times of cooling form gelatin aggregates and primary emulsion breaks. These samples (2M and 2N) were not completely gelled, big gelatin aggregates were observed macroscopically instead. Manual agitation facilitated thermic homogeneity and formed less aggregation structures. Stopping the agitation for a short period of time (<30s) led to non-uniform gelation. Some of the images obtained after cooling the samples are shown in Figure 8.3.

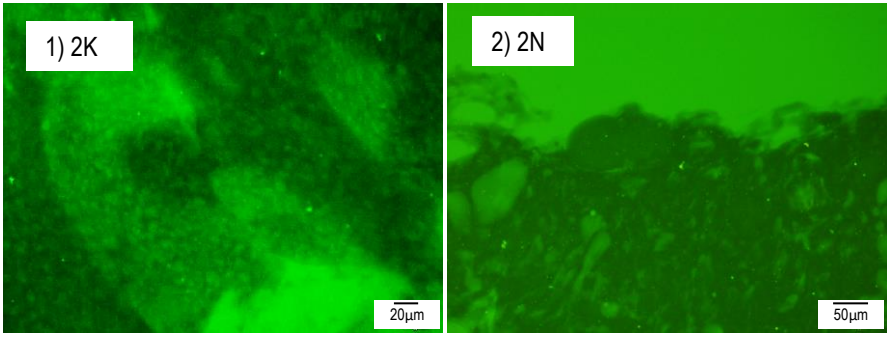


Figure 8.3. Images observed after cooling in an ice bath. The conditions are given in Table 8.1. Maltodextrin is labelled with FITC.

9. CROSSLINKING WITH GENIPIN

Emulsion stabilization by cooling was not achieved so chemical crosslinking with genipin was also tested. Genipin was introduced either in primary emulsion or in multiple emulsion. A previous study of gelation time with different gelatin solutions, which had the same concentrations as used in emulsions preparation, was performed.

9.1. GENIPIN IN PRIMARY EMULSION

Genipin was added at different steps, of simple emulsion formation, as it is shown in Table 9.1.

Table 9.1. Concentrations of genipin and description of the method of addition. Al samples were 5% M and 12 %G. PT: Phase transition. HEM: High energy method.

Sample	Genipin (wt%)	Addition	Method
1E	0.8	With maltodextrin before pH change	PT
1F	0.4	With water after pH change	PT
1G	0.4	With maltodextrin stock solution	HEM
1H	0.8	With maltodextrin stock solution	HEM

Genipin added before pH change in phase transition method (Sample 1E) lead to non-uniform gelation as crosslinking is affected by the pH of solution. When sodium hydroxide powder was added, instantly it was observed that gelatin become a dark gel. However, a M/G primary emulsion was obtained by addition of genipin after pH increase (Sample 1F). Primary emulsions obtained by this method and with Ultraturrax (Samples 1G and 1H) were studied along time in a water bath at 50°C. In all cases, the apparent size of maltodextrin primary droplets seems to decrease with time until complete gelation (Figure 9.1). It occurs after 12 minutes in emulsions with 0.8% genipin and after 20 minutes with 0.4%. As was observed in previous experiments, maltodextrin droplets break while gelatin gelation. In conclusion, gelation time needs to be long enough to allow ME preparation but preventing their destabilization.

In all cases, the blue colour appeared in the sample as time elapsed. As explained in the Introduction, oxygen induces a radical polymerization of genipin and forms a blue coloured gel in presence of gelatin.

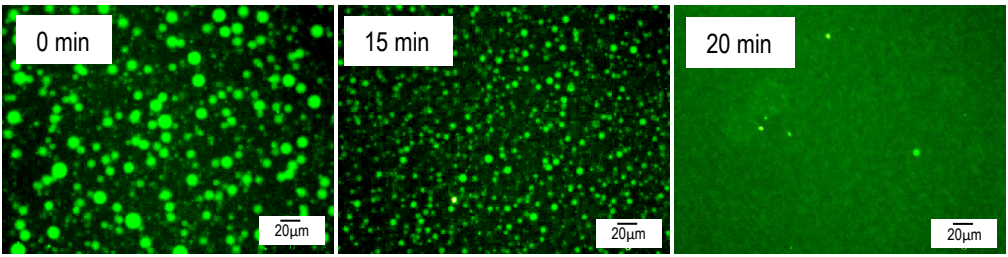


Figure 9.1. Emulsion 2P (Table 8.1) along time at 50°C. Maltodextrin is labelled with FITC.

9.1. GENIPIN IN MULTIPLE EMULSION

Genipin was added with the outer maltodextrin phase in the concentrations indicated in Table 9.2. The blue gel was formed in the upper part of the sample. Genipin crosslinks the gelatin, forming a blue viscous layer, which eventually gellifies (Figure 9.2). In order to reduce this layer formation, different additional stirring conditions were applied after emulsion preparation.

Table 9.2. ME prepared at different conditions with genipin.

Sample	wt% Genipin	Extra stirring time (min)	Stirring speed (rpm)
2O	*0.1*	*1*	*100*
2P	*0.2*	*1*	*100*
2Q	*0.2*	*10*	*100*
2R	*0.4*	-	-
2S	*0.4*	*5*	*200*
2T	*0.4*	*10* *5*	*150* *100*
2V	*0.8*	*2*	*150*

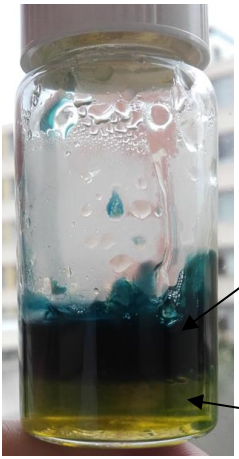


Figure 9.2. Multiple emulsion 2Q after 30minutes.

High genipin concentration (sample 2V) increases viscosity and does not allow ME formation. A weight percentage lower than 0.2% has long gelation times and the formation of the blue layer is higher than in emulsions with concentrations about 0.4%. The longer time, the darker blue gel is formed. As seen before, strong agitation increases coalescence and creaming, but emulsion breakdown also occurs in absence of agitation. Low speed agitation was applied. Sample 2S, agitated 5 minutes at 200 rpm, had the thinnest blue layer on the surface.

In terms of the stability, some multiple emulsions droplets remain in the lower phase minutes after the preparation. Figure 9.3 shows the crosslinking of gelatin along time. Initially a multiple emulsion is formed by the procedure explained before. While the top blue layer is becoming a gel, it is difficult to extract a fraction from the clarified region, located at the bottom of the vial. The specimen observed in the microscope contains traces of the upper gel with a filament-like

appearance. After gelation in the upper part of sample, some multiple emulsion droplets remained in the maltodextrin solution.

Very different droplet sizes were observed, form 20µm to 200µm diameter. These droplets were not uniformly distributed through the maltodextrin phase. Aggregates were also formed but with less number of droplets than those observed in previous experiments.

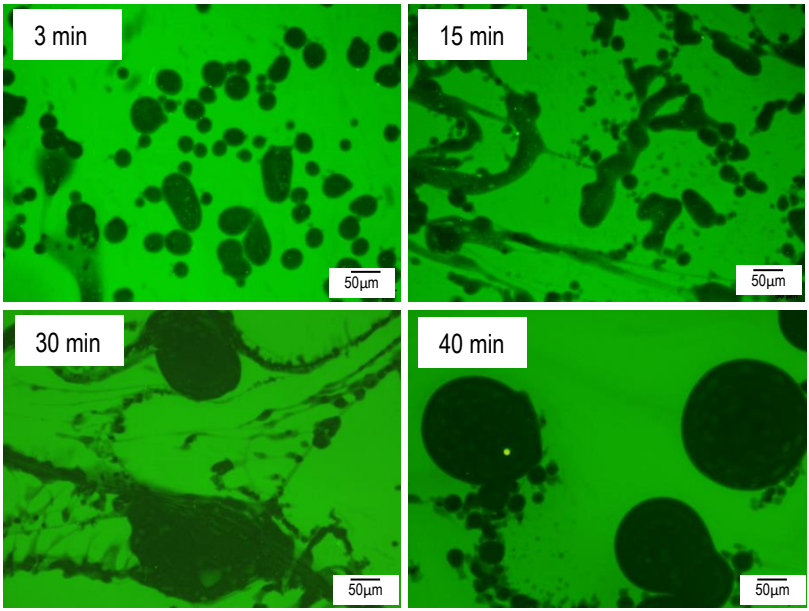


Figure 9.3. Multiple emulsion 2P (Table 9.2) along time, at 50°C water bath, of specimens extracted from the bottom of the vial. Maltodextrin is labelled with FITC.

Inner maltodextrin droplets are not completely spherical in shape, probably gelation breaks them, as observed before. However, droplet break is slow and many multiple structures are visible after 40 min (Figure 9.3).

Although some multiple emulsion structures have been obtained by this method, encapsulation yield would be very low. Almost all gelatin in the solution stays in the upper blue gel. Therefore, formation of multiple M/G/M emulsions was tried by another method, based on changing temperature.

10. MULTIPLE EMULSIONS FORMATION BY TEMPERATURE CHANGE

Previous work in the research group found by accident another method to form multiple emulsions [9]. Simple emulsions gelatin-in-maltodextrin were prepared in compositions close to the binodal line. By cooling down these emulsions, a secondary phase separation occurred, and internal maltodextrin droplets formed spontaneously inside gelatin droplets. Emulsions were prepared at 50°C and the phase separation was induced by decreasing the temperature.

The intersection of the binodal line and the tie line determines the composition inside the initial gelatin droplets of simple G/M emulsions. As the overall composition is close to the binodal line, the gelatin dispersed phase will contain a high percentage of maltodextrin. The G/M droplets act as an independent reservoir which might go thought another phase separation. In this case, temperature change induces a displacement of the binodal line and promotes formation of internal droplets. This is due to oversaturation of maltodextrin inside gelatin droplets, and this might not occur if compositions are distant from the binodal line [9].

This separation was achieved in a composition of 12% M and 4%G and it was observed in the microscope operated with differential interference contrast. There was also a separation in the composition of 13%M and 4%G. The temperature was controlled with a hot stage with different cooling rates. Either by lowering the temperature 2°C/min or 4°C/min, phase separation started at 30°C (Figure 10.1).

In all cases, smaller gelatin droplets were also present and no phase separation was appreciated there. The multiple M/G/M emulsion droplets seems to be quite stable.

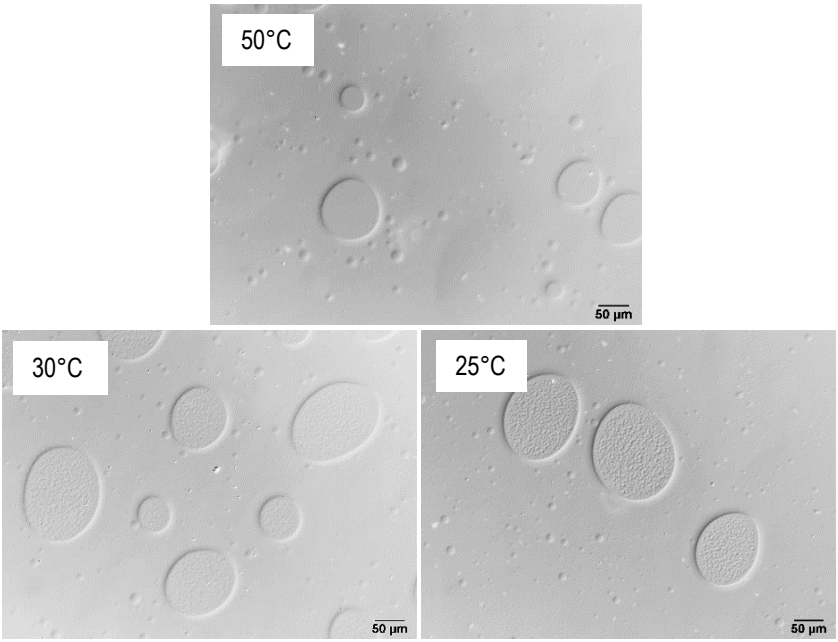


Figure 10.1. Emulsion with 13%M and 4%G, at different temperatures. Cooling rate: 2°C/min.

In order to obtain stabilized multiple emulsions, the sample was cooled down after its preparation in an ice bath for 60 or 10 seconds, and subsequently, diluted with 16g of cold water. As it is shown in Figure 10.2, some multiple emulsions droplets remain in the sample in big aggregates. These droplets are smaller than those observed at 50°C.

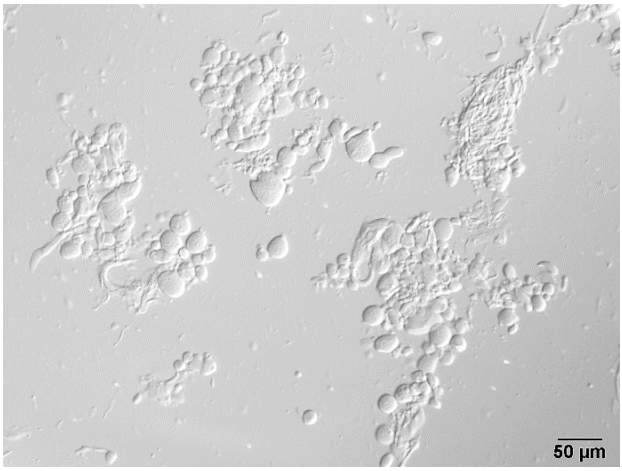


Figure 10.2. Emulsion 13%M and 4%G after cooling and diluting.

11. CONCLUSIONS

Simple W/W emulsions

- Both simple M/G and G/M emulsions can be prepared either by high agitation or by phase transition methods.
- Smaller M/G droplets can be obtained by a phase transition based on increasing pH. In all cases, G/M droplets are bigger than M/G droplets, probably because of the higher viscosity of gelatin aqueous solutions.

Preparation and properties of Multiple W/W/W emulsions, by a two-step method.

- The optimal conditions were as follows:
  a. The primary emulsion contained 12% Gelatin and 5% Maltodextrin, and it was prepared by the phase transition method.
  b. The external solution of multiple emulsion was prepared with 30% maltodextrin.
  c. The multiple M/G/M was prepared by dropwise addition of primary emulsion to the external solution, at 50/50 mixing ratio and 250 rpm, for 3 min at 50ºC.
- Stabilization was not achieved by decreasing temperature. Coalescence and creaming occurred producing emulsion breakdown. Inner primary maltodextrin droplets were also not very stable.
- Chemical crosslinking of gelatin, with genipin, led to formation of microgels with multiple structure. However, they were highly polydisperse, heterogeneous and with low stability.

Preparation and properties of Multiple W/W/W emulsions, by changing temperature.

- More stable multiple M/G/M emulsions were successfully obtained by changing temperature of simple W/W emulsions with compositions near to the binodal line. These M/G/M emulsions were formed thanks to a secondary phase separation.

12. FUTURE WORK

Regarding the high biocompatibiliy and low cost, water-in-water-in-water emulsions composed by gelatin and maltodextrin would be very useful in pharmaceutical applications for encapsulated systems. For future work it would be interesting:

- To stabilize multiple emulsions droplets obtained by temperature change with genipin. And subsequently, to separate these droplets by freeze-drying process.
- To stabilize multiple emulsion droplets with nanoparticles (Pickering emulsions).
- To study the encapsulation and release of active components in the inner primary maltodextrin droplets.

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14. ACRONYMS

ATPS: Aqueous Two-Phase Systems.

CP: Continuous phase.

DIC: Differential interference contrast.

DMSO: Dimethyl sulfoxide.

FITC: Fluorescein isothiocyanate.

G/M: gelatin-in-maltodextrin.

G: Gelatin.

HEM: High energy method.

M/G/M: maltodextrin-in-gelatin-in-maltodextrin.

M/G: maltodextrin-in-gelatin.

M: Maltodextrin.

ME: Multiple emulsion.

PE: Primary emulsion.

PT: Phase transition.

RBITC: Rhodamine B isothiocyanate.

W/W/W: water-in-water-in-water.

W/W: water-in-water.

APPENDICES

APPENDIX 1: DIFFUSION STUDY

In order to study the diffusion across the different regions of multiple emulsions, two fluorescent labelling agents were used, FITC (green) and RBITC (red). Maltodextrin in primary emulsion was labelled with FITC, and the outer phase was labelled with RBITC. After preparation of M/G/M multiple emulsion, the sample was observed in the microscope using two different colour filters (Figure A1.1).

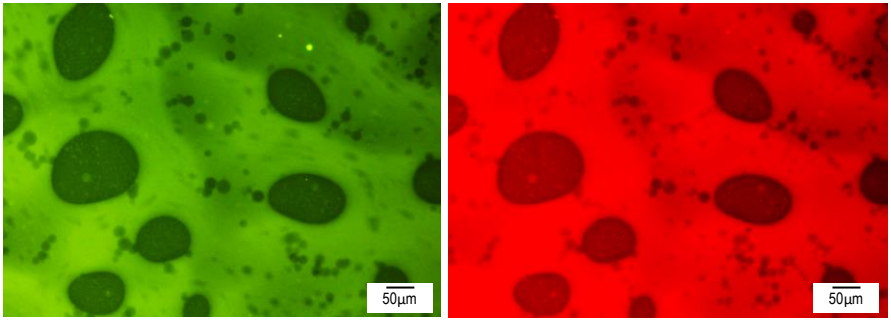

Figure A1.1. Sample where primary emulsion maltodextrin is labelled with FITC and outer maltodextrin with RBITC. A) Image obtained wit FITC filter. B) Image obtained with RBITC filter.

In both images, inner and outer maltodextrin phases are coloured which means that diffusion occurs in both directions. However, images obtained in later experiments showed contradictory results. Some samples labelled with only one of the agents showed fluorescence using both filters. Other samples labelled with both fluorescein and rhodamine produced different shades. It is not known if these results were due to lack of fully monochromatic filters in the microscope. Thus, the results observed cannot not be conclusive, and should be ascertain using a more specialized fluorescence microscope.