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Grau de Química

Treball Final de Grau

Novel biocompatible microgels for functional food, obtained in water-in-water emulsions.

Nous microgels biocompatibles per aliments funcionals, obtinguts en emulsions de tipus aigua-en-aigua (W/W).

Cristina Miquel Espigulé

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Campus d'Excel·lència Internacional
BKC Barcelona Knowledge Campus

“Solo existen dos días en el año en que no se puede hacer nada. Uno se llama ayer y el otro mañana.”

Dalai Lama

En primer lloc, m’agradaria agraïr a l’Insitut de Química Avançada de Catalunya (IQAC), al Consell Superior d’Investigacions Científiques (CSIC) i, en concret, al Dr. Jordi Esquena Moret, per poder formar part del seu grup de recerca i dur a terme la realització del meu treball final de grau. També m’agradaria donar les gràcies a la Dra. Maria Sarret Pons per donar-me la oportunitat de veure el dinamisme del món empresarial en el major organisme públic d’investigació d’Espanya.

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I per últim, però no menys important, m’agradaria expressar una inmensa gratitud cap als meus pares, que gràcies al seu esforç i vocació, puc escriure aquesta memòria.

REPORT

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1. SUMMARY

The aim of this study is to prepare microgels obtained from water-in-water (W/W) emulsions, which could be used as a drug delivery vehicle of different active ingredients. Microgels are colloidal dispersions of cross-linked gel particles with the ability to swell in response to a change of physicochemical parameters (pH, temperature, ionic strength, etc.). In the last few years, microgels have been investigated due to their biocompatibility, their high water content and their use of biocompatible polymers such as proteins and polysaccharides. Moreover, their controlled drug release at the target site improves the drug efficacy and reduces side effects.

The (W/W) emulsions have been formed by a dispersion of an aqueous phase into another aqueous solution. Two mutually immiscible hydrophilic polymers have been used. As other emulsions, (W/W) emulsions are no thermodynamically stable.

In the present work, the phase behaviour of the system Carboxymethylcellulose (CMC) – Bovine serum albumin (BSA) mixtures has been was studied at two different pH conditions: neutral and basic. At neutral pH, coacervates were formed and it was not possible to obtain emulsions. However, at basic pH, an immiscibility region with coexistence of the aqueous phases was detected. The respective phase diagram could be drawn and water-in-water (W/W) emulsions were prepared. The emulsion droplets were relatively stable and could be examined under the optical microscope. They presented instability by coalescence, which was reduced by decreasing pH. Emulsions behaviour, which were composed of CMC droplets neutralized with acid, was studied. CMC gels have been cross-linked with Fe^{3+} ions, and capsules and beads, made of cross-linked CMC, have been obtained.

Keywords: microgel, water-in-water emulsion, active ingredient, colloidal dispersion, biocompatibility, superficial tension, instability, miscibility, phase separation.

2. RESUM

L'objectiu d'aquest treball és obtenir microgels a partir de emulsions aigua-en-aigua que es podrien utilitzar com a mitjà d'encapsulació i alliberament controlat de diferents principis actius. Els microgels es defineixen com dispersions col·loïdals de partícules de hidrogel, constituïdes per un polímer hidrofílic entrecreuat, que tenen la propietat de reaccionar enfront estímuls externs (pH, temperatura, força iònica, etc.) variant la seva mida. Han esdevingut objecte d'estudi gràcies a la seva elevada biocompatibilitat, el seu gran contingut en aigua i la utilització de polímers com ara proteïnes, polisacàrids, i altres macromolècules biocompatibles.

En el present treball, els microgels s'han obtingut utilitzant emulsions de tipus aigua-en-aigua com a plantilla, per controlar la mida de partícula dels microgels. Les emulsions aigua-en-aigua estan formades per una dispersió d'un medi aquós dintre d'un altre medi aquós. Estan formades per la mescla de dos polímers hidrofílics immiscibles entre ells. Com totes les emulsions, les aigua-en-aigua són termodinàmicament inestables.

S'ha estudiat el comportament fàsic del sistema Carboximetilcel·lulosa (CMC) – Albúmina de sèrum boví (BSA), a dos pH diferents: neutre i bàsic. A pH neutre es forma un coacervat que precipita, i no és possible obtenir emulsions. En canvi, a pH bàsic s'obté una regió d'immiscibilitat, amb coexistència de dues fases aquoses. S'ha determinat el diagrama de fases, i s'han obtingut emulsions de tipus aigua-en-aigua (W/W). Les gotes d'emulsió són relativament estables i s'han observat al microscopi òptic. Presentaven inestabilitat per coalescència, que es va reduir disminuint el pH. S'ha analitzat el comportament de les emulsions, constituïdes per gotes de solució de CMC neutralitzada amb àcid. Posteriorment s'ha estudiat l'entrecreuament de gels de CMC amb Fe^{3+}, i s'han obtingut càpsules i partícules sòlides de CMC entrecreuat.

Paraules clau: microgel, emulsió aigua-en-aigua, principi actiu, dispersió col·loïdal, biocompatibilitat, tensió superficial, inestabilitat, miscibilitat, separació de fases.

3. INTRODUCTION

One of the most accurate and complete definitions of *Colloid* is the following: "A colloid is a dispersed or polyphase system of one or more phase dispersed of particles with a dispersed phase distributed through a continuous phase." The size of a colloidal particle is between 1 nm y 1 μm [1]. Thomas Graham, the pioneer in colloidal chemistry, coined on 1861 the term Colloid to describe "pseudo-solutions" [2]. The term "Colloid" comes from the Greek term "κόλλα", which means "glue". Depending on the division level of the particles, colloids can be classified in three groups [3]:

- **Association colloids:** a grouping of amphiphilic molecules. They are hydrophilic/lipophilic and thermodynamically instable systems.
- **Colloidal dispersions:** they are formed by particles with no (or a little) affinity to the dispersant medium. Colloidal dispersions are lyophobic/hydrophobic and thermodynamically instable systems.
- **Solution of macromolecules:** the dispersed molecules are molecules or ions. They are hydrophilic/lipophilic and thermodynamically stable systems.

On the other hand, colloidal dispersions can be classified as [4]:

- Solid dispersions (solids, gels and solid foams)
- Liquid dispersions (sols, emulsions and foams)
- Gaseous dispersions (aerosols and liquid aerosols)

3.1. GELS

The word "gel" comes from the term "Gelu" in Latin, which means frozen or immobile [5]. According to IUPAC [6], the definition of gel is the following: "*A gel is a nonfluid colloidal network or polymer network that is expanded throughout its whole volume by a fluid*." Gels flow under shear have both elastic (solid-like) and viscous (liquid-like) components. Consequently, properties of gels are between those of solids and liquids. Gels can be classified according to different criterions. Based on the nature of the continuous phase they can be named as:

- Hydrogels: the solvent is water.
- Organogels: the solvent is an organic solvent.

3.2. HYDROGELS

Hydrogels are usually defined as 3-Dimensional networks of hydrophilic polymers. The hydrophilic structure of hydrogels renders them capable of holding large amounts of water in their structure (they can contain over 90% of water). Their absorption capacity of water is due to the presence of hydrophilic groups such as -OH, -COOH, -CONH or $-SO_3H$ within the polymer [7, 8, 9].

In order to stabilize hydrogels in solution, cross-linking is required to fix the hydrogel structure. A crosslinker is a substance that creates a bond which links one polymer (can be synthetic or natural) chain to another. These bonds may be covalent or ionic bonds (Figure 1).

If a crosslinker is used, interactions between the polymer chains are enforced and the polymer adopts a fixed conformation and it is more difficult to be disrupted with solvent changes. As a consequence, the hydrogels are more stable after cross-linking. These stable hydrogels can have swelling/deswelling as a function of external stimuli. For instance, at basic pH, carboxylate groups of the polymer will be charged leading to higher electrostatic repulsion between the polymer chains, and thus, to swelling of the gel.

There are several types of cross-linking gels methods [10, 11]:

- Chemical cross-linking: the crosslinker creates covalent links with groups of the polymer chains. Various examples are cross-linking by radical polymerization, cross-linking by chemical reaction of complementary groups, cross-linking using enzymes…etc.
- Physical cross-linking: the crosslinker creates ionic junction with groups of the polymer chains. Various examples are as cross-linking by ionic interactions, cross-linking by hydrogen bonds or cross-linking by protein interactions.

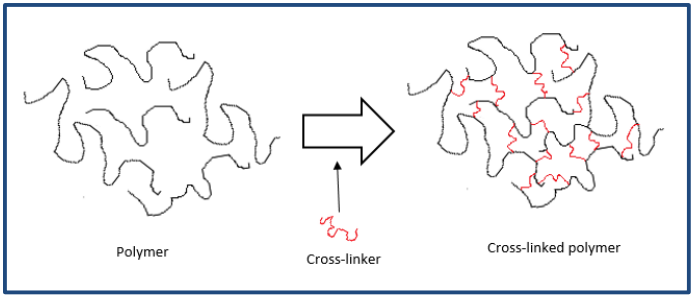


Figure 1. Illustration of the cross-linking process.

Hydrogels have many applications. Among the most important, one could name the following [7, 10, 12]:

- Wound dressing
- Sanitary pads
- Trans-dermal delivery systems
- Materials for dental implants

A very important novel application of hydrogels is their use in drug delivery. Controlled and targeted release of drugs is a challenge for traditional drug administration. For that reason, for several decades different types of hydrogels have been investigated to resolve those challenges.

3.3. MICROGELS

Microgels are colloidal dispersions of cross-linked gel particles with the ability to swell in response to a change of physicochemical parameters. In the last few years, microgels have been investigated to use them as drug delivery vehicles due to their capacity to incorporate and release molecules and active ingredients. The term “microgel” was introduced in 1949 by Baker, who referred to cross-linked polybutadiene latex particles [13, 14].

Microgels can be very appropriate as drug delivery systems because they combine the aspects of colloidal dispersions with the ones of macrogels. They have a high surface to volume ratio, which facilitates mass transport to and from the microgels, but also display controlled swelling, which makes them responsive delivery vehicles. Moreover, their controlled drug release at the target site improves the drug efficacy and reduces side effects. The drug loading

is relatively high and it can be achieved without chemical reactions, preserving in this way its activity[15].

Therefore, microgels offer some advantages that include the protection from hydrolysis and other kinds of chemical and enzymatic degradation in addition to the toxicity reduction.

There are many different methods for microgel preparation such as Atomisation, Injection/Extrusion, Spray drying, Spray cooling, Solvent desorption, Emulsion-templating, Microfluidics, Membrane emulsification or Shear gels [16, 17]. The present work is focused on biopolymer-based microgels produced by water-in-water emulsions, as it will be explained in the following section.

Microgels have been used, for many years, in a wide variety of different applications (Surface coating, printing, oral care products, food products, etc.) [18, 19, 20], and in the last few years, microgels have started to be studied as novel drug delivery vehicles [19].

3.3.1. Responsiveness of microgels

Microgels can be responsive to different physicochemical parameters, which means that they may swell or deswell as a response to external changes. It is known that hydrogels are sensitive to: Temperature, pH, ionic strength, osmotic pressure and others.

As a response to these changes, the active component can be released through swelling, fragmentation or erosion of the microgel or by the diffusion (Figure 2).

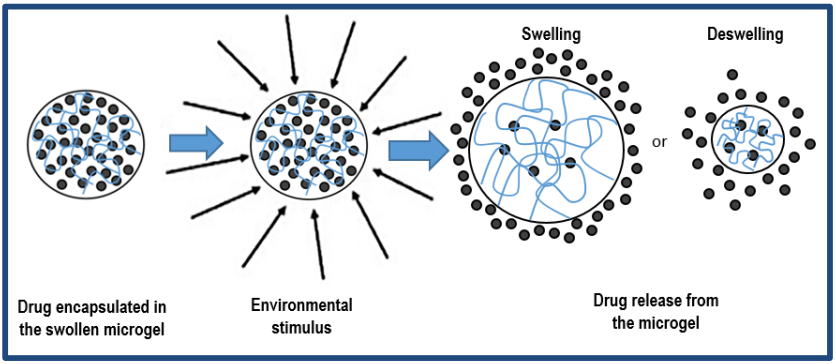


Figure 2. Schematic representation of a microgel as a drug delivery vehicle.

3.3.1.1. Temperature-responsiveness of microgels

The hydration of the polymer changes due to a change in temperature, and as a consequence of this swelling or deswelling take place. The active component can be either released from the capsule in the swollen state (dissolving out mechanism), or by the "squeezing out" mechanism, in which the drug is released during deswelling [19, 21], as shown in Figure 2.

3.3.1.2. pH-responsiveness of microgels

As well as with temperature, the solubility of the microgel changes due to a change of the pH in the environment, and as a consequence it swells or deswells. Considering that the intention is to encapsulate the drug into the microgel, which has to pass through the stomach to reach the intestine, it is hoped that it would be stable at acid pH (stomach region) and that its solubility would rise at basic pH (intestine region) [22]. Hence, if the cross-linked polymer from which the microgel is produced is stable at acid pH (insoluble and no swelling), will remain stable at the stomach because its groups will be protonated and there will not be electrostatic interactions between the polymer and the charges in the stomach. When it will arrive at the gut, the pH there is basic, so as the pH will be higher that the pKa, the groups of the molecule will be charged, and there will be electrostatic repulsion between them and it will open its structure. As a consequence, its pores will be bigger and the liquid will enter into it so the biopolymer will swell and dissolve finally.

3.4. EMULSIONS

3.4.1. Definition and main aspects

Becher (1965) [23] and Everett (1972) [24] defined emulsions as follows: *"An emulsion is a heterogenic instable thermodynamically system, which is formed by, at least two liquids, and immiscible among them, phases, whom one of them is dispersed (dispersed or intern phase) into the other one (extern or scattered phase) under the guise of little droplets, the diameter from which if, in general, above 0.1 μm. This system has a minimum stability, which can rise through the use of stabilizers agents or surfactants."*

A way to classify emulsions is according to the size of droplets in the dispersed phase:

- **Macroemulsions:** above 1 μm.
- **Nanoemulsions:** between 20 and 200 nm.

Another way to order emulsions is depending on the nature of the dispersed and the continuous phase, according to which emulsions can be classified in simple and multiple emulsions. They will be described below [25, 26]:

Simple emulsions are categorized as:

- Oil-in-water (O/W)
- Water-in-oil (W/O)
- Oil-in-oil (O/O)
- Water-in-water (W/W)

3.4.2. (W/O) and (O/W) emulsions

When water droplets are dispersed in oil the emulsion formed is called water-in-oil emulsion and vice versa, if oil droplets are dispersed in water the emulsion formed is called oil-in-water emulsion. What determines whether the emulsion formed will be one type or another is the type of emulsifier that will be used and the concentration of both phases [27].

Such as (W/O) and (O/W) emulsions experience a phase separation due to the immiscibility of the hydrophobic character of oil and the hydrophilic one of water due to the presence of polar groups in the hydrophilic phase and the non-polar in the hydrophobic ones.

3.4.3. (O/O) emulsions

These emulsions are composed of two immiscible oils, one of which forms the droplets of dispersed phase and the other constitutes the continuous phase. (O/O) emulsions can be prepared using combinations of two immiscible oils, for example, dispersions of hydrocarbons and fluorocarbons, or a highly hydrophobic hydrocarbon dispersed within a polar hydrocarbon. Some authors mention that (O/O) emulsions can be used either as an active ingredient delivery vehicle [28].

3.4.4. (W/W) emulsions

Water-in-water emulsions are a new type of simple emulsions formed by a dispersion of an aqueous phase into another aqueous solution. Water-in-water emulsions can be formed by the use of two mutually immiscible hydrophilic polymers (Figure 3).

It is known that these emulsions can be prepared by using a combination of two mutually immiscible hydrophilic polymers. Beijerinck first noted, in 1896, the 'incompatibility' of certain polymers in aqueous solution [29]. This behaviour is rather common in mixtures of proteins and polysaccharides, due to their high immiscibility [30, 31].

Some known examples of water-in-water emulsions can be found in mixtures of dextran and polyethylene glycol (PEG), Starch and PEG, gelatine and dextran, casein and amylopectin, among others [30]. An example of a large emulsion drop is shown in Figure 3.

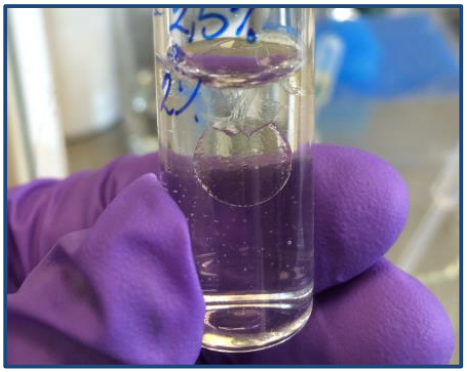


Figure 3. Example of a water-in-water emulsion, prepared by adding a solution of CMC into another solution of BSA.

(W/W) emulsions have some advantages respecting to (W/O) and (O/W) emulsions as that they do not use oil components, they have low interfacial tension and that they are biocompatible, facts that make them interesting in medical applications. However, a possible drawback is that their stability is not often high.

3.4.5. Multiple emulsions

These sorts of emulsions are characterised in that the droplets of their dispersed phase contain at the same time droplets inside which are not miscible with the droplet and that are miscible with the continuous phase. They are classified in [32]:

- Water-in-oil-in-water (W/O/W)
- Oil-in-water-in-oil (O/W/O)

The structure of simple (O/W) and (W/O), multiple (W/O/W) and (O/W/O) emulsions are shown in Figure 4.

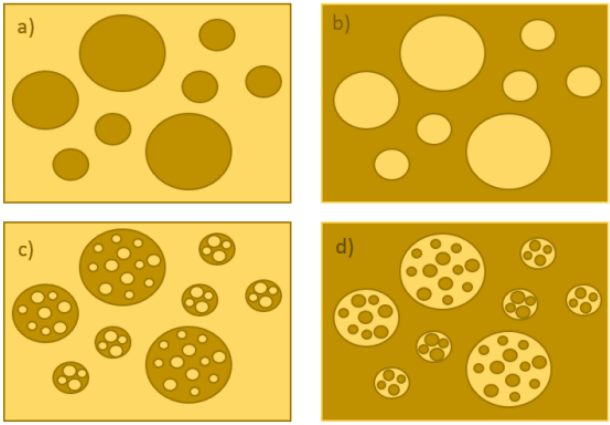


Figure 4. Examples of different types of emulsions: (a) oil-in-water (O/W); (b) water-in-oil (W/O); (c) multiple water-in-oil-in-water emulsion (W/O/W); (d) multiple oil-in-water-in-oil (O/W/O).

3.4.6. Emulsion stability

As mentioned before, emulsions are no thermodynamically stable. Therefore, emulsions have kinetic stability, and phase separation might occur over time due to several breakdown processes. In several of the breakdown processes it is difficult to find the exact factors as they may take place simultaneously.

The main mechanisms are: creaming (or sedimentation), flocculation, coalescence and Ostwald Ripening, as described in the Figure 5 [31, 33, 34, 35, 36].

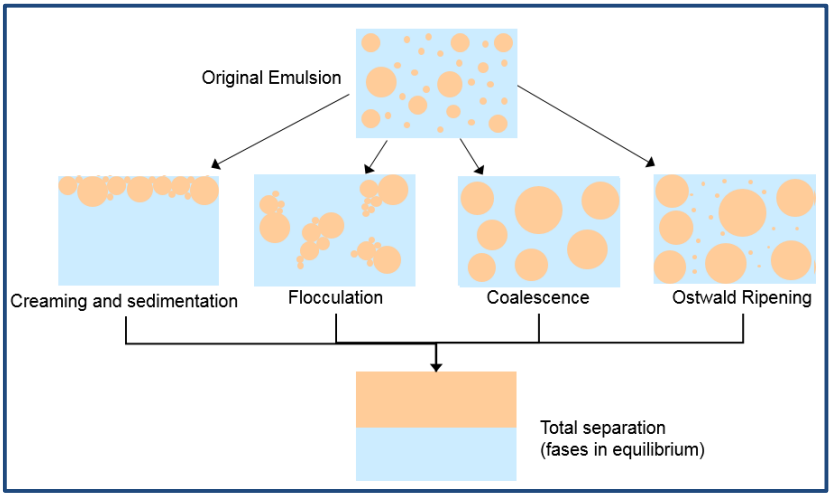


Figure 5. Scheme that shows the various breakdown processes that lead to emulsions rupture.

- Creaming and sedimentation: This process results from external forces, such as gravitational or centrifugal. When such forces exceed the thermal motion of the droplets (Brownian motion), a concentration gradient builds up in the system with the larger droplets moving faster to the top (if their density is lower than that of the medium) of to the bottom (if their density is larger than that of the medium).
- Flocculation: Aggregation of droplets into larger units due to attraction forces. It occurs when there is no sufficient repulsion between the droplets which would repel them from each other.
- Coalescence: Coalescence is the process of thinning and disruption of the liquid film between the droplets with the result of fusion of two or more droplets into larger ones. With time, this leads to complete separation of the emulsion into two distinct liquid phases. The driving force for coalescence is the film fluctuations.
- Ostwald Ripening: This process origins from the finite solubility of the liquid phases. Liquids that are referred to as being immiscible often have mutual solubility. The smaller droplets have larger solubility compared to the larger ones. Thus with time, the smaller droplets become merged on the larger droplets leading to a larger amount of big droplets.

3.5. COLLOIDAL DISPERSIONS IN MIXTURES OF HYDROPHILIC POLYMERS

The behaviour of polymer mixtures in water is very complex. The interactions forces between two hydrophilic polymers can be either attractive of repulsive. These interaction forces between polymers and the different hydration forces with water will determine the macroscopic behaviour.

When two solutions of hydrophilic polymers are mixed, depending on the thermodynamics of mixing, four different cases can occur [37] depending if the interaction forces are attractive or repulsive:

1. **Attractive forces between hydrophilic polymers:**
   - Soluble complexation: in this case, the two polymers form a complex because of the attractive interactions between them. This complex might be soluble, often due to excess of electrostatic charges and/or strong hydration. A solution of complexed molecules appear, and one stable liquid phase is observed.
   - Associative phase separation: it is induced by strong attractive forces between the two polymers, leading to the formation of a coacervate or precipitate. In this case, the polymers might sediment, and the excess water is expelled as a supernatant.
2. **Repulsive forces between hydrophilic polymers**
   - Segregative phase separation: The phenomenon occurs when the two hydrophilic polymers are incompatible, and formation of two separated phases is observed.
   - Complete miscibility (ideal mixed solution): It is due to weak interactions between the components of the system. In this case, all the components are co-solubilized in one single macroscopic phase.

These four different cases are shown, schematically in Figure 6.

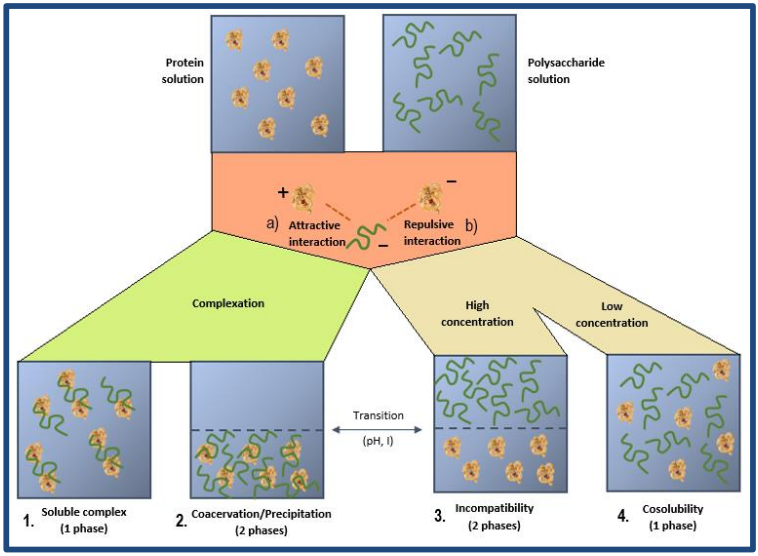


Figure 6. Possible interactions in a mixture of two biopolymers.

The phenomena described here depends on both the thermodynamic and kinetic aspects of mixing. These aspects determine the phase behaviour of biopolymer mixed solutions. Primarily, the phase behaviour of two solutes in solution depends on the sign of the free energy of mixing, which is calculated from ΔH_{mix} and ΔS_{mix}, according to the Gibbs free energy of mixing.

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} \qquad \textbf{Equation 1}$$

If ΔGmix is positive, the solution mixture will tend to form two different phases, whereas negative ΔG_{mix} produces a mixing of the two polymers. ΔH_{mix} usually is positive, so it favours the demixing of two solutes in solution. However, the value of ΔG_{mix} strongly depends on the entropy term ΔS_{mix}. In most cases of solutes with low molecular weight, ΔS_{mix} is large (and positive) so that the two solutes mix. However, often it does not occur with polymeric solutes, in which molecular conformation is restricted by the presence of another large molecule. In these cases, ΔS_{mix} is much smaller than ΔH_{mix} and the two solutes demix. The phase separation is observed because the two polymers cannot occupy the same volume due to their physical dimensions or charge characteristics. This reduces the entropy of mixing and thus ΔG_{mix}

increases [27, 37]. In addition, above a critical concentration, an osmotic force also favours separation of the system into two immiscible phases, each rich in one of polymers [16]. The formation of two separated phases depends on the sign and the degree of electrostatic charge on the two polymers. Certainly, segregative phase separation will never occur when the two polymer have opposite charges, which would produce a strong attraction. Segregative phase separation can be observed when at least, one of the polymers is non-ionic [16, 38].

3.5.1. Formation of (W/W) emulsions by segregative phase separation

This case, in which two immiscible aqueous solutions are formed, is the most interesting for the present work. Water-in-water emulsions can be prepared because of segregative phase separation. The two aqueous solutions can form emulsions, in which the dispersed phase consists of a solution of the first polymer, and the continuous phase consists of the second polymer. In the present work, water-in-water emulsions are used to obtain microgels, and thus, segregative phase separation is explained in more detail.

In systems composed of water and a mixture of two hydrophilic polymers, a region of immiscibility, in which two aqueous phases coexists, may appear. Ionic strength and pH can influence the system by changing it from an associative to a segregative phase separation. The phase behaviour of mixed polymer solutions is often studied using composition phase diagrams at constant temperature.

Phase diagrams indicate the two different regions areas, a region (two phase region) where the free energy of mixing is positive, thus 2 phases coexist, and the other (one phase region) where the free energy is negative, thereby solutions of one phase are formed [37, 38, 39]. A scheme of typical phase behaviour is shown in Figure 7.

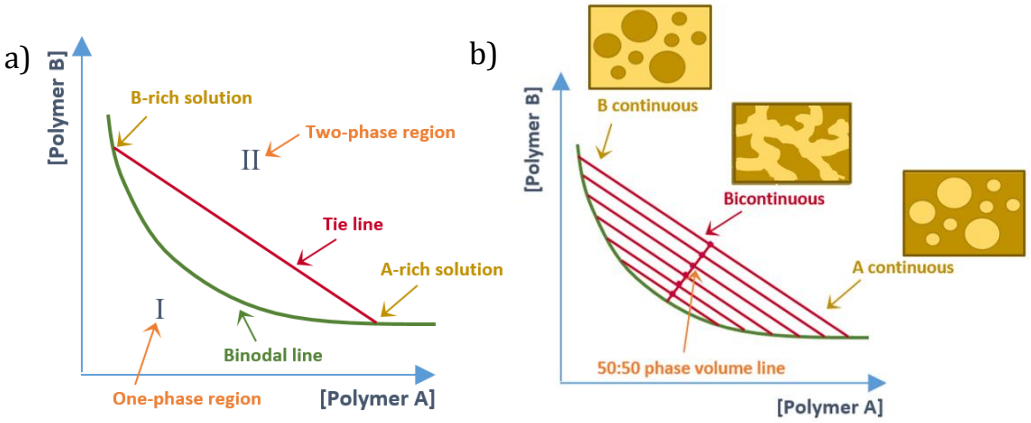


Figure 7. Representation of a (a) binary phase diagram and (b) propperties of the mixture solution in function of the polymer concentration.

The line that represents the boundary between the one-phase and two-phase regions of the phase diagram is the binodal line. Each single point of the phase diagram represents a mixed biopolymer solution in terms of the fractional concentrations of both polymers. If the point is in the two-phase region of the phase diagram, the system will show phase separation, in which the compositions of the two separated phases are described by the end-points of the binodal line [37].

The ratio between the relative lengths of the two sections on the tie-line, gives the information of the relative volume of the two separated phases. In the upper-left regions of the phase diagram (Figure 7 b), where the overall composition is rich in polymer B, emulsions composed of droplets that are enriched in polymer A, surrounded by a continuous solution that contains most of polymer B, are formed. In the opposite case, when concentration of polymer A is higher, the droplets contain most of polymer B, and are surrounded by a continuous solution which contains most of polymer A. In intermediate situations, bicontinous emulsions can be formed.

3.5.2. Biopolymer selection

The aim of this study is to prepare microgels obtained from water-in-water emulsions. These emulsions are formed by the mixture of two polymers, in this case biopolymers. Biopolymers in

the dispersed phase, forming later the microgels, should be stable at acid pH. Furthermore, a pair of polymers which are incompatible and can form under some conditions phase separation had to be found.

For these reasons, as a polysaccharide, Carboxymethylcellulose (CMC) was selected because it is an acid-stable polymer and it is viscous. Its pKa is 4.3. On the other hand, from the list of proteins, the Bovine Serum Albumin (BSA) was selected due to its incompatibility (phase separation) with CMC. The polymer is zwitterionic with an isoelectric point (pI) of 4.7.

Another possibility for the protein could be the AluProt-CGNA (Centro de Genómica Nutricional Agroacuícola), isolated from Lupin plant extract [40].

4. OBJECTIVES

The main objective of this work is the obtaining and study of microgels, as a novel drug delivery system, which could encapsulate an active ingredient. Considering that the microgels are aimed to be used as drug delivery for oral administration, the microgels should be stable at acid pH, and thus the active component would be delivered in the intestine. These microgels should be made of biocompatible polymers, such as polysaccharides and proteins, in order to avoid the creation of toxic products in the body as a result of an undesired side reactions.

A second important objective is to study the use of water-in-water (W/W) emulsions, based on biopolymer mixtures, as reaction media for the preparation of the microgels. These emulsions can be formed in aqueous mixtures of two hydrophilic polymers, which can be biocompatible macromolecules. (W/W) emulsions will be investigated due to they do not use surfactants or oil (and it avoids any purification) and above all, it is a novel system which have been started to be used recently.

Therefore, polymers that are biocompatible macromolecules will be the focus of this work. The ideal candidates are polysaccharides and proteins, which can form (W/W) emulsions. An interesting combination of two biopolymers can be mixtures or Carboxymethylcellulose (CMC) and Bovine serum albumin (BSA), because of the following reasons:

- CMC: it is anionic polysaccharide, derived from cellulose.

- BSA: is an albumin protein derived from bovine serum.

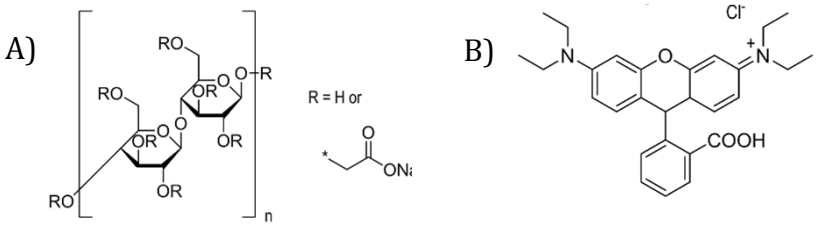
It is known that mixtures of CMC and BSA lead to segregative phase separation [30] that can allow to prepare (W/W) emulsions.

Moreover, a third important objective is to study the response of microgels particles, as a function of external stimuli. For this reason, their swelling and deswelling behaviour depending on pH will be examined. CMC microgels will be studied as well, considering that it is an interesting biopolymer that exhibits sensitivity to changes of pH. It remains stable at acid pH and it dissolves at basic pH due to a growth of its solubility.

5. EXPERIMENTAL SECTION

5.1. MATERIALS

1. Sodium carboxymethylcellulose: Molecular weight (MW): 250 kDa. (Sigma-Aldrich, ref.: 1001361695, CAS: 9004-32-4). **1.**
2. Bovine Serum Albumin: MW: 66.5 kDa. Heat shock fraction, pH=7, assay: ≥ 98 % form: lyophilized powder. (Sigma-Aldrich, ref.:1002028817, CAS: 9048-46-8).
3. Hydrochloric acid fuming 37%: for analysis EMSURE® ACS, ISO, Reag. Ph Eur.. (Merck Millipore, ref.: 1003171000, CAS: 7647-01-0).
4. Sodium hydroxide: BioXtra, ≥ 98 %, pellets (anhydrous). MW: 40 g/mol (Sigma-Aldrich, ref. 101204018, CAS: 1310-73-2).
5. Filtered deionized water: Milli-Q® water. Deionized water filtered by the ultra-pure Millipore water system, model Synergy Smart UV (resistivity at 25ºC: 18.2 MΩ·cm; conductivity 0.056 µS/cm, water quality: type I, ion concentration < 1µg/L.
6. Rhodamine B: MW: 479.02 g/mol (Sigma-Aldrich, ref. R6626, CAS: 81-88-9). **2.**
7. Lupin variety AluProt-CGNA isolated from Lupin seeds (Lupinus liteus, a legume plant): it has a purity of 97.54 g protein/100 g (CGNA, Chile).
8. $FeCl_3$: MW: 162.20 g/mol, reagent grade, 97%, hygroscopic (Sigma-Aldrich, ref.: 157740-100G, CAS: 7705-08-0).



1 **2**

Figure 8. Formulas of A) Carboxymethylcellulose **1.** and B) Rhodamine B **2.**

5.2. EQUIPMENT AND INSTRUMENTAL

1. Optical Microscope: Olympus model BX51TRF-6, coupled to a digital camera Olympus DP73, controlled with an image/video capture software Stream Essential of Olympus.
2. pH meter: Mettler Toledo, model Seven Easy.
3. Thermostated Bath: a 15 L of water bath of methacrylate with temperature controlled by HAAKE DC10 thermostat.
4. Ultraturrax: Janke & Kunkel, IKA-Labortechnik Stauten, model T25, dispersing element S25N-10G.
5. Dry-bath: P-Selecta Multiplaces.
6. Overhead stirrer: Heidolph, model RZR 2041.
7. Vortex: IKA® VORTEX GENIUS 3.
8. Centrifuge: Eppendorf model 5804R, maximal velocity 5000 revolutions per minute (rpm) with a maximal working temrperature of 40ºC.
9. Analytical balance: Mettler Toledo AB204-S/FACT balance with a precision of $\pm 10^{-4}$ g (maximum capacity: 220 g).
10. Balance: Sartorius CPA3202-S top-loading balance with a precision of $\pm 10^{-2}$ g (maximum capacity: 3200 g).
11. Heater: KOTTERMANN 2712 heating and drying oven (maximum temperature: 250 ºC).
12. Magnetic stirrer: JP Selecta Model: Multimatic-5N

5.3. METHODOLOGY

5.3.1. Preparation of the solutions

The 3% (W/W) CMC stock solutions were prepared by dispersing CMC into water Milli-Q and then stirred during 4 hours at 25ºC due to its high viscosity. The same process was carried out for the 5% (W/W) CMC. The final volume was always completed with water Milli-Q.

5.3.2. Study of swelling of CMC (untreated and neutralized/heated) at different pH

5.3.2.1. Preparation of CMC stock solution

The 15% (W/W) CMC stock solution was prepared by dispersing CMC into water Milli-Q and then stirred during a whole day at 25ºC, due to its high viscosity. The viscosity was so high that the overhead stirrer was required for dissolving all the polymer.

5.3.2.2. Determination of swelling degree

CMC was:

- Neutralized with acid/heat treatment: HCl 1 M was added at the dissolution and the samples were heated at 80 ºC during 3 hours.

- Cross-linked with Fe^{3+} ions: the solution of CMC was added inside a solution of $FeCl_3$ 2% [41, 42, 43].

Three small pieces of each sample were cut and immersed into three vials of 5 mL of HCl 0.1 M, NaOH 0.1 M and H_2O Milli-Q, as it is shown in the following image (Figure 9).

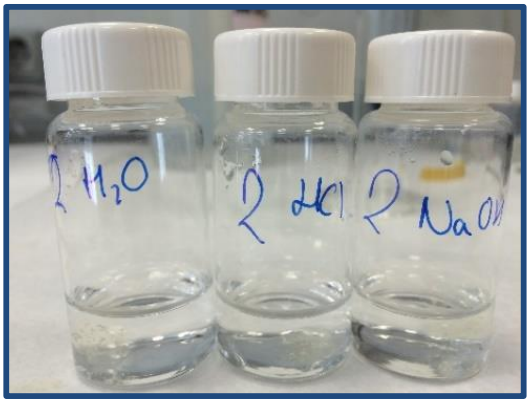


Figure 9. Three small pieces of CMC untreated inside of each vial of different solutions (pH=2, pH=6 and pH=8).

After 3, 6, 9, 12 min….until hours after, the gel was removed from the solution, blotted dry with a tissue and weighted. In the cases where the gel was too liquid or broke when it was took with the spatula, experiments were not continued. The same procedure was carried out for the neutralized CMC, as shown in Figure 10.

Figure 10. Representation of the swelling effect of neutralized CMC in solution.

The swelling index was calculated as follows:

$$\text{Swelling Index} = \frac{m_t - m_0}{m_0} \cdot 100\ \% \qquad \textbf{Equation 2}$$

m_t is the mass of the gel at different time points and m_0 the mass of the gels at initial time.

Three little piece of gels (previously weighted) were introduced inside three vials, separately, which one was at pH=2, and the others at the pH=6 and pH=8. After having introduced those pieces, and controlling the time, weight was measured each 2 or 3 minutes. Thus, later, was possible to estimate the swelling of them.

5.3.3. Determination of phase diagrams

The binodal line of this system was determined to know exactly at which concentrations of CMC and BSA, two phases were present, and as a consequence, (W/W) emulsions could be formed.

These experiments were performed at two different pH conditions.

5.3.3.1. Neutral pH

5.3.3.1.1. Preparation of the mixtures

The 3% (W/W) CMC stock solutions were prepared by dispersing CMC into water Milli-Q and then stirred during 4 hours at 25ºC due to its high viscosity. As the 5% (W/W) resulted to be so viscous, it was chosen the 3% in order to assure the appropriate mixture of both biopolymers. The final volume was always completed with water.

The 25% (W/W) BSA stock solutions were prepared by dispersing BSA into water Milli-Q and then stirred during 1 hour at 25ºC, it is not as viscous as CMC. The final volume was always completed with water.

5.3.3.1.2. Identifying continuous/dispersed phase

1 mg/mL (W/W) (very dilute due to its high colour capacity) Rhodamine B was added into the sample of 1/4, after vortexing the sample during 10 s, was left. Rhodamine B is a fluorescent dye with a λ_{ex}= 554 nm and a λ_{em}= 627 nm at basic pH [44].

5.3.3.1.3. Observation of phase behaviour

The samples, once prepared were vortexed during 10 seconds and then introduced into the thermostated bath at 25 ºC. As soon the samples equilibrated (between one and two days), phase behaviour was observed by eye.

Mixtures of CMC and BSA at different concentrations were prepared in order to determine their phase behaviour. CMC concentrations ranging from 0 - 2.5 % (W/W) and BSA concentrations from 0 - 8 % (W/W) were examined. Mixtures will be denoted from now on as % CMC / % BSA. For instance, 1/4 is 1 % CMC / 4 % BSA.

5.3.3.2. Basic pH

5.3.3.2.1. Preparation of the mixtures

The 3% (W/W) CMC stock solutions were prepared by dispersing CMC into NaOH 0.1 M and then stirred during 4 hours at 25ºC. The final volume was always completed with NaOH 0.1 M solution.

The 25% (W/W) BSA stock solutions were prepared by dispersing BSA into NaOH 0.1 M and then stirred during 1 hour at 25ºC. The final volume was always completed with NaOH 0.1 M solution.

5.3.3.2.2. Identifying continuous/dispersed phase

1 mg/mL (W/W) (very dilute due to its high colour capacity) Rhodamine B was added into the sample of 1/4, after vortexing the sample during 10 s, was left to stabilize and after that, was verified if Rhodamine B dyed more the CMC than BSA due to that it was more negatively charged (even more than in neutral pH).

5.3.3.2.3. Observation of phase behaviour

It was followed the same procedure as the phase diagram at neutral pH, but in this case at basic pH. Mixtures of CMC and BSA at different concentrations were prepared in order to determine their phase behaviour.

CMC concentrations ranging from 0 - 2.5 % (W/W) and BSA concentrations from 0 - 8 % (W/W) were examined. The samples, once prepared were vortexed during 10 seconds and then placed into the thermostated bath at 25 ºC. As soon as the samples reached equilibrium (it last between one and two days), phase behaviour was observed by eye (Figure 11).

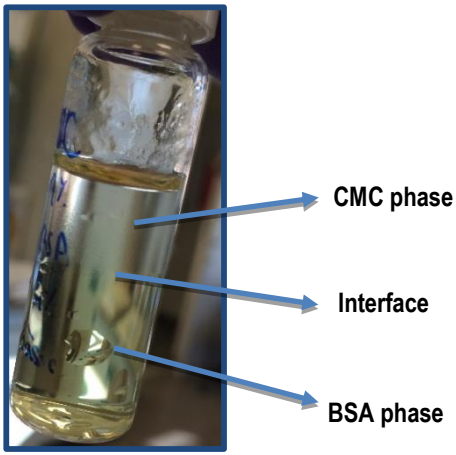


Figure 11. Photograph of a sample showing an example of phase separation

There was studied the influence of temperature and pH on phase diagram, to see if those vials where the gelification was so high that it blocked the phase separation, increased their fluency and if it was a change on the situation of the binodal line. The vials were placed at 50 ºC in a thermostated bath and their behaviour was studied at neutral and at basic pH.

5.3.4. Evaluation of emulsion stability

Experiments were performed to evaluate the possibility of formation of droplets of CMC into the BSA medium. To see this phenomenon, some droplets of several emulsions were examined on the microscope.

After having checked that the emulsions were stable, there were chosen those with a clear phase separation and with a % of BSA higher that CMC (it is known that the higher volume of a substance makes it the continuous phase) due to it is interested to find droplets (dispersed phase) of CMC into the BSA continuous phase.

For microscopic images, the samples were vortexed during 10 seconds and a droplet was placed on a glass slide and observed under the microscope at room temperature. The first sample analysed was 1/8 in neutral pH and after having completed the phase diagram in basic pH, the first sample analysed, after having vortexed it during 10 seconds, was 1/4. The same procedure was performed but the sample was this time stirred with Ultraturrax.

After that, it was analysed the emulsion 2.5/2.8 for its moderate viscosity. A small volume of HCl 0.1M was added to the emulsion and immediately it was stirred with Ultraturrax and examined under the microscopy. Other emulsions with similar concentrations of CMC/BSA were also studied, as 2.25/2.8, 2.5/2.6 and 2/2.8.

5.3.5. Centrifugation

After having checked the presence of droplets in the sample, it was centrifuged, first, during 10 min at 2000 rpm and after, at 4000 rpm during the same time as before.

5.3.6. CMC cross-linked with Fe^{3+} ions

The 3% CMC stock solution was cross-linked with a solution of $FeCl_3$ 2%.

The 2% $FeCl_3$ solution was prepared by dispersing $FeCl_3$ into water Milli-Q and then stirred during a few minutes at 25ºC.

Once having cross-linked the solution of CMC, it was studied its pH dependence, placing three different jelly beads of it in three different vials, one with a dissolution of pH=2 (HCl), and the others with pH=6 (Milli-Q water) and pH=8 (NaOH). Then the bead introduced in the HCl vial, was introduced after at the NaOH vial.

6. RESULTS AND DISCUSSION

6.1. PRELIMINARY STUDIES ON PROTEIN EXTRACTED FROM LUPINUS LUTEUS

These preliminary studies were performed to evaluate whether the protein could be used for the formation of hydrogels and microgels. Therefore, the gelification of the protein was studied, first, at three concentrations: 12, 16 and 20% of protein. The solutions were dissolved at pH=7 and NaOH was added to reach pH=9. Then, they were stirred until completely dissolution (overnight). After that, they were transferred into test tubes and heated for 1 hour in a dry bath at 100 ºC, followed by rapid cooling in cold water bath. Then, the test tubes were further cooled at 4ºC for 2h. It was observed the formation of a gel at the 16 and 20% solutions, but there was not gel formation at the 12% solution, it was rather a precipitate, as it is shown in the Figure 12.

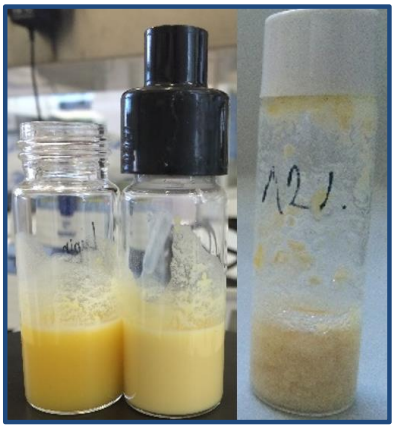


Figure 12. Photograph of three samples of protein extracted from Lupinus Luteus at 20, 16 and 12% concentrations in water, respectively.

It was observed that gels were only formed using rather high concentrations. Then, it was concluded that the protein extract was not useful for the present work. Another protein, BSA, was selected.

6.2. PHASE BEHAVIOUR OF CMC-BSA MIXTURES

Phase diagrams of CMC-BSA mixtures were studied for two different pH: neutral (pH~7) and basic (pH~13) at 25 ºC. Figure 13 displays the phase diagram obtained at pH=13. It was assumed that the densest phase (observed at the bottom of test tubes) is the BSA-rich phase due to its more yellowish appearance.

The binodal line separates the one-phase region from two-phase region, in the phase diagram. Below the binodal line, at lower polymer concentrations, only one liquid phase was observed, in which both polymers were soluble. Above this line, at high polymer concentrations, two immiscible phases coexist, as indicated in Figure 13.

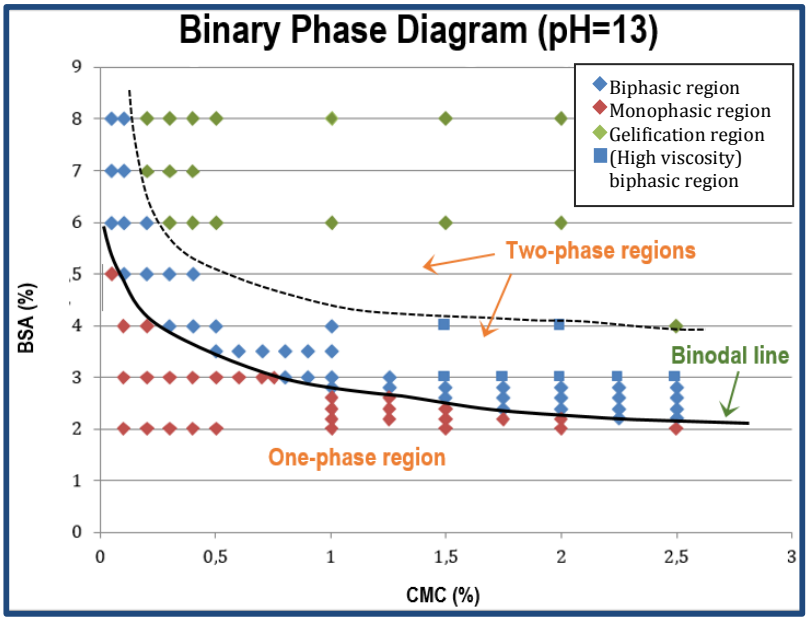


Figure 13. Binary phase diagram at pH=13 of the CMC/BSA aqueous mixtures

As it is said in the previous section, the first phase diagram done was at neutral pH, but most of the solutions did not show a clear phase separation, they showed rather the formation of a coacervate. For this reason, the pH was changed to basic pH. At this pH it was possible to draw a phase diagram because no coacervates were formed in vials, and a clear phase separation was observed.

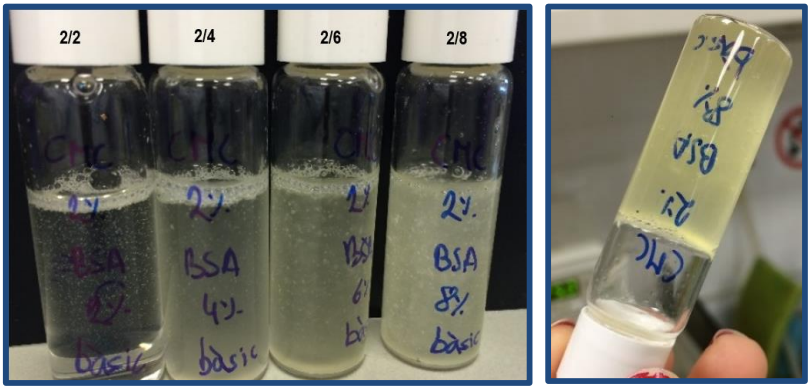


Figure 14. Representation of the viscosity rise due to the BSA concentration rise in solution (picture on the left) and the no evidence of fluency at solutions with high concentration of BSA (gel formation) at basic pH (picture on the right). The compositions (CMC/BSA concentration ratios) are indicated.

Moreover, the emulsion became a gel at high concentrations of BSA. These concentrations are indicated in Figure 13, in green colour. The higher was the BSA concentration, the more viscous was the mixture (Figure 14), until reaching a point where the mixture did not have any fluency now that it formed a gel. In addition, the gelification was so rapid that the system did not have enough time to stabilize, so there was no possibility to see any phase separation on the points where it was supposed to appear.

This may have occurred because of denaturation of BSA. This phenomenon causes that the protein (in this case BSA) loses its molecular conformation, which is present in their native state, because of the reduction in pH. BSA loses thus its native compact structure to a more lineal one. This allows its chains to entangle, leading to strong interactions and forming a gel.

6.3. IDENTIFICATION OF CONTINUOUS/DISPERSED PHASE

To distinguish the two phases in the polymer mixture, the cationic dye Rhodamine B was added to the mixture of 1/4 (where there was a phase separation), expecting that there would be stronger interaction with the CMC-rich phase due to the anionic character of this phase. However no increased colouring of either phase was observed. Rhodamine B homogenously distributed into both phases of BSA and CMC. It was tried at solutions of neutral and basic pH, and the same result was obtained: all the liquid phases had the same colour.

As Rhodamine B did not provide information, each phase was identified by measuring qualitatively the relative volume fraction of each phase, and relating it to the composition. It was considered that the phase with larger volume would correspond to the polymer with higher concentration.

6.4. OBTAINING OF EMULSIONS FROM TWO-PHASE SYSTEMS

The mixtures of 1/8, 1/4, 2.5/2.8, 2.25/2.8, 2.25/2.6, 2.5/2.6 and 2/2.8 (CMC-BSA concentration ratios), all with phase separation, were vortexed and observed under the microscope at room temperature. The first composition examined was 1/8, at neutral pH, and droplets were not observed under the microscope. The second composition studied was 1/4, this one at basic pH, and the presence of droplets was observed. This emulsion was prepared by an initial soft agitation with vortex, and a stronger agitation with Ultraturrax at 2500 rpm. However, the droplets were not stable, and the droplet size increased with time, as shown in Figure 15, which displays an emulsion, observed soon after preparation ($t \approx 0$) and the same emulsion after 10 min, in which the droplets are larger. Coalescence was visible under the optical microscope, producing an increase of droplet size as a function of time.

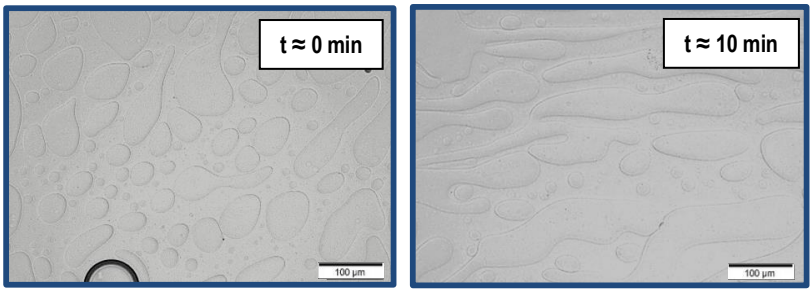


Figure 15. Microscope images of the 1/4 emulsion at pH=13, showing the high coalescence of the emulsion (left) and the formation of large droplets after 10 min (right).

It was expected that an increase in viscosity could reduce coalescence. For this reason, experiments were performed adding a small volume of 0.1 M HCl, which decreased pH from 13 to 12. This small pH change, increased the viscosity of the emulsion, and indeed, it became more stable. This emulsion was observed under the microscope (Figure 16)

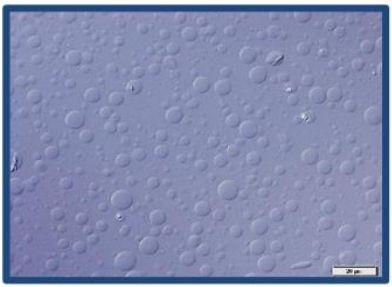


Figure 16. An example of microscopy image that shows a water-in-water emulsion, consisting of CMC-rich droplets dispersed in BSA-rich solutions: 1/4 sample at pH 12, after adding a small amount of HCl.

This result, obtained using a composition with CMC/BSA ratio of 1/4, clearly indicated that a small decrease in pH could greatly increase emulsion stability. Consequently, adjustment to pH 12 was performed in all further experiments. The same results were observed at higher polymer concentrations: 2.5/2.8, 2.5/2.6, 2.25/2.6, 2.25/2.8 (CMC/BSA ratios). Examples are shown in Figure 17.

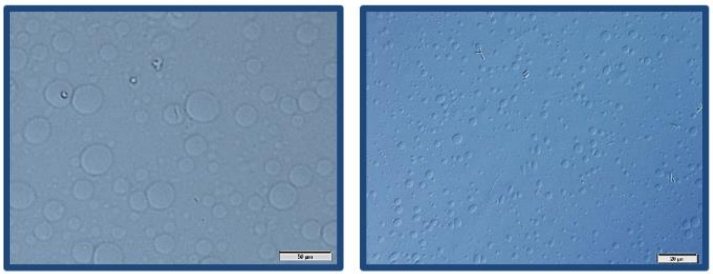


Figure 17. . Two examples of microscopy images that show water-in-water emulsions, consisting of CMC-rich droplets dispersed in BSA-rich solutions: 2.5/2.8 (left) and 2.5/2.6 (right) at pH=12. These emulsions were observed one week after being prepared, without centrifugation.

The above images clearly show stable water-in-water (W/W) emulsions, since no significant changes in droplet size were observed for a period of one week. In fact, the droplets remain so stable that samples could stand up centrifugation. The emulsions were centrifuged, first at 2000 and then 4000 rpm, for 10 minutes each. However, no sedimentation was observed. It can be attributed to several reasons:

- Emulsions are quite transparent, because of refractive index matching between the dispersed and the continuous phase. Therefore, the sediment can be difficult to be observed under the naked eye.

- Emulsions are more stable, and phase separation is prevented. Most likely, the addition of a small amount of HCl could increase the degree of denaturalization of BSA protein, increasing the viscosity of the external phase of the emulsion. This increase in viscosity might enhance emulsion stability.

In any case, one can conclude that stable (W/W) emulsions had been obtained. The stability against centrifugation is illustrated in Figure 18, which shows an emulsion, observed under the optical microscope, after being centrifuged.

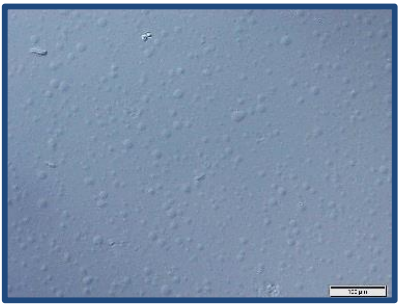


Figure 18. An example of microscopy images that show water-in-water emulsions, consisting of CMC-rich droplets dispersed in BSA-rich solutions: 2.5/2.6 sample at pH 12, after centrifugation, at both 2000 rpm (10 min) and 4000 rpm (10 min).

6.5. GELIFICATION OF THE CMC DROPLETS OF EMULSIONS

6.5.1. Preliminary studies

After emulsion formation, the possible methods to gelify the CMC droplets were evaluated, in order to obtain microgel particles. Two CMC solutions, at 3 and 5% concentrations, were prepared. Afterwards, their response against temperature and pH changes was studied. Due to its high viscosity, the 5% CMC solution was almost a gel, with a low fluency.

- Temperature effect: Temperature was increased to approximately 50ºC, and then cooled down to 0ºC. It was observed that viscosity was lower at high temperature, and increased after cooling down, as expected.
- pH effect: Both 0.1 M HCl and 0.1 M NaOH were added. As expected, viscosity increased after adding NaOH, because of ionization of carboxylate functional groups of the CMC.

In any case, to form a hydrogel, cross-linking was required. For this purpose, Fe^{3+} was tested as a crosslinker, since it is a simple and biocompatible method.

6.5.2. Formation of capsules by cross-linking CMC with Fe^{3+}

CMC was cross-linked with Fe^{3+} ions, evaluating the possibilities of capsule formation. First, solutions of 0.01%, 0.05%, 0.1 % and 1% of $FeCl_3$ were added to CMC solutions. The concentration of the CMC solutions were the same as those previously studied (0.5, 1, 1.5, and 2 wt% CMC). Small volumes of the $FeCl_3$ solutions were added to the CMC solutions, and the mixture was agitated using a vortex stirrer. A precipitate was formed almost immediately, which sedimented after a few hours. Capsules were not observed in this precipitate. The formation of the precipitate was observed in all studied concentrations, and the amount of precipitate depended on the amount of both CMC and iron. It was also performed with an emulsion of CMC/BSA mixture. The scheme of this process is shown in Figure 19.

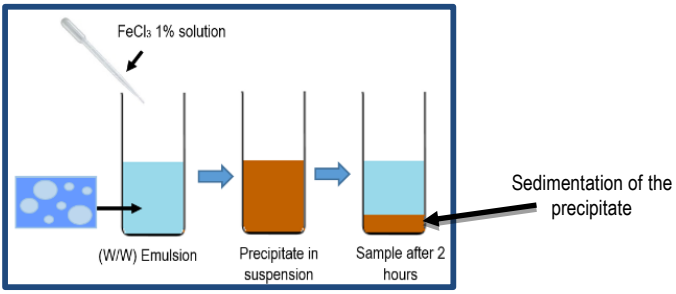


Figure 19. Squematic representation of cross-linking a (W/W) emulsion of CMC/BSA. Only cross-links the emulsions in contact to the crosslinker and a thus precipitate is formed.

The formation of the precipitate was attributed to the complexation of carboxylate functional groups with iron ions, which occurred very rapidly and without control. For this reason, it was suggested that mixing could be more controlled by inverting the order of addition: Adding the emulsion on the iron solution, instead of adding the iron solution on top of the emulsion.

It was attempted to find a concentration where the CMC cross-linked but the BSA not. BSA concentrations (those used in the phase diagram) with 0.01%, 0.05%, 0.1 % and 1% of $FeCl_3$ were cross-linked with $FeCl_3$ to see if there was a concentration where BSA did not cross-link. However all concentrations cross-linked.

Another procedure was tested for the formation of capsules. An emulsion with 2.5/2.8 was added to $FeCl_3$ solutions, at 1 and 2wt% (Figure 20). The emulsions were introduced inside the $FeCl_3$ solution and large capsules were formed (image shown in Figure 20). It was observed that the capsules contained a liquid, but gelification occurred within minutes inside the capsules. This was attributed to the diffusion of iron ions inside the capsules.

A capsule was removed just after preparation and cut, in order to observe the capsule interior. Emulsion droplets were not observed under the microscope.

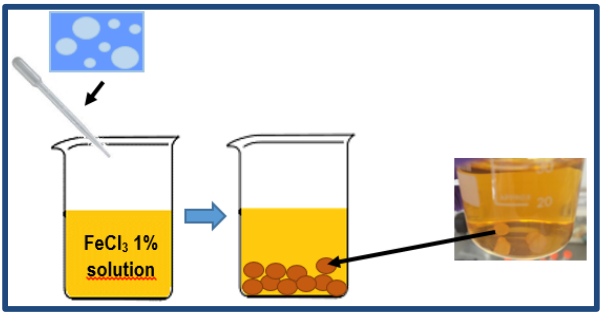


Figure 20. Squematic representation of cross-linking a (W/W) emulsion of CMC/BSA by introducing it inside a $FeCl_3$ 1% solution.

In order to control better the process, and study the cross-linking of CMC with Fe^{3+}, the influence of pH was studied in more detail, as described in the following section.

6.6. SWELLING CMC STUDIES

The influence of pH was studied by measuring the swelling of CMC over a period of three weeks, first in absence of Fe^{3+}. CMC gels were prepared, at 15wt% concentration, were placed in contact with three aqueous solutions. The pH of these aqueous solutions was either 2, 6 or 8. The weight of the CMC gels was measured as a function of time, as described in the experimental section. The results of swelling are shown in the followings tables (Table 1 and Table 2). Table 1 displays the results obtained for CMC without any further treatment (untreated CMC gel), whereas Table 2 shows those results obtained for CMC gels that were neutralized with HCl (treated CMC gels), and partially dried at 80ºC for three hours.

Similar experiments were performed with CMC gels, previously cross-linked with Fe^{3+}. These capsules or gels were rather rigid and did not show any swelling. Therefore, results with

presence of iron salt are not shown in the following tables. However, the properties of the capsules will be described in detail in section 6.7.

Untreated CMC gel (15wt% CMC)					
pH= 2		pH=6		pH=8	
Time [min]	Swelling [%]	Time [min]	Swelling [%]	Time [min]	Swelling [%]
0	0	0	0	0	0
1	0,897	1	140	1	182
4	1,05	4	191	4	340
7	1,09	7	203	7	359
10	1,26	10	231	10	418
15	1,32	15	285	15	It has dissolved
45	1,40	45	It has dissolved	45	It has dissolved
75	1,67	75	It has dissolved	75	It has dissolved
105	It has dissolved	105	It has dissolved	105	It has dissolved
135	It has dissolved	135	It has dissolved	135	It has dissolved

Table 1. Data of the swelling behavior of CMC untreated, as a function of the pH of the media.

Treated CMC gels					
pH= 2		pH=6		pH=8	
Time [min]	Swelling [%]	Time [min]	Swelling [%]	Time [min]	Swelling [%]
0	0	0	0	0	0
1	420	1	358	1	465
4	530	4	616	4	1479
7	698	7	782	7	1657
10	731	10	950	10	2044
15	821	15	1304	15	2171
45	847	45	1584	45	It has dissolved
75	856	75	1751	75	It has dissolved
105	861	105	1836	105	It has dissolved
135	863	135	It has dissolved	135	It has dissolved

Table 2. Data of swelling of CMC gels neutralized with an acid and post-treated at 80ºC, as a function of the pH of the media.

The data included in the tables was plotted as a function of time (Figures 21 and 22).

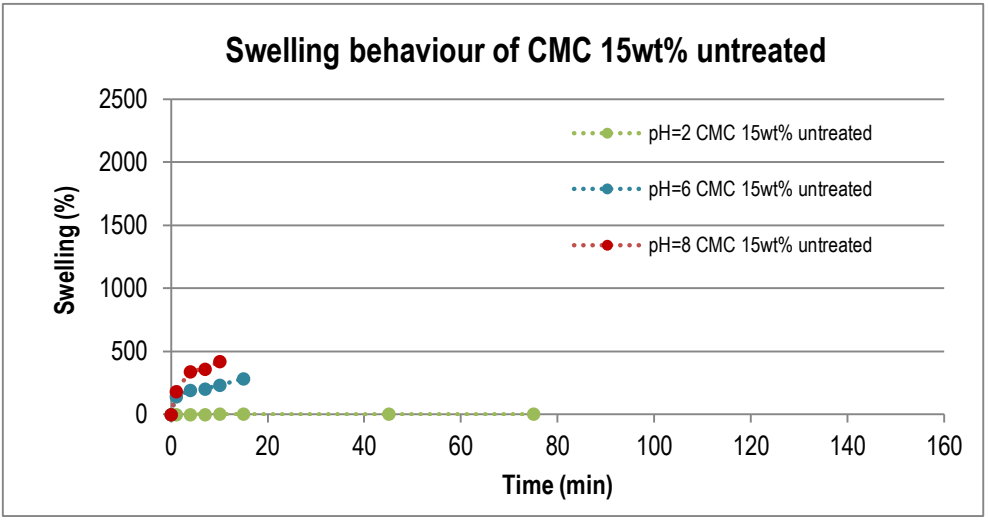


Figure 21. Swelling behaviour of untreated CMC gels as a function of time for different pH values of the external solutions.

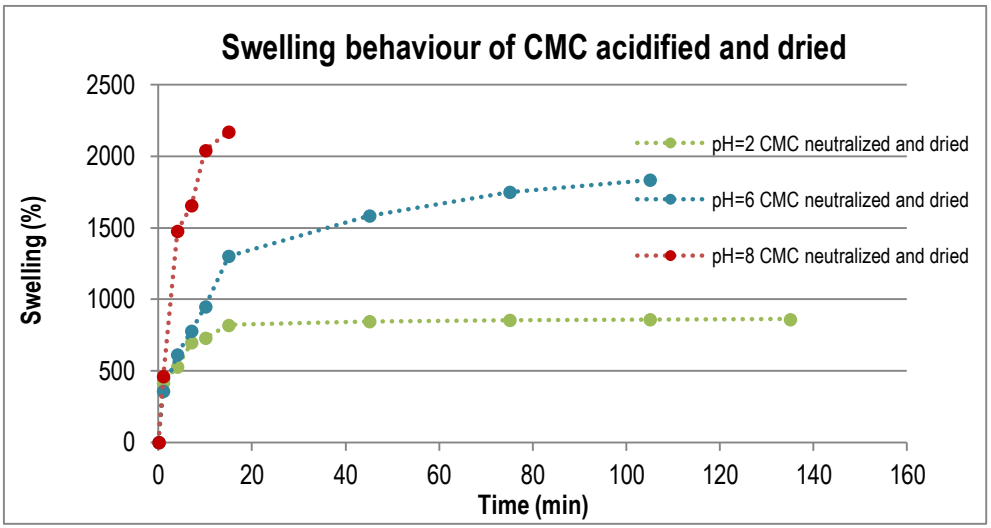


Figure 22. Swelling behaviour of CMC gels, neutralized with HCl and dried, as a function of time for different pH values of the external solutions.

The results shown in Figures 20 and 21 (also shown in Tables 1 and 2) clearly confirmed the behaviour of CMC dependent on pH. CMC is more soluble at higher pH than at lower pH, and therefore, swelling increases with pH. The higher pH value, the larger swelling ratio will be. The higher level of swelling at pH=8 is probably due to the free charges of CMC at this pH. The pKa of CMC is 4.3. At pH above 4.3, the carboxylic groups in the polymer get charged which leads to an electrostatic repulsion between charged chains. Therefore, this allows more solvent penetrating the gel, and leading to an increase in swelling. At pH=6, this value is still higher than the pKa of the polymer, and thus, a large degree of swelling is also observed.

At pH=2, in contrast to the situation at pH=8, there is almost no swelling. The untreated gel show almost no swelling, and the dried gel reaches a rather constant value in only 15 minutes. In this sample, swelling does not increase further in several weeks (not shown in the graphic). This fact can be due to the neutral charge of CMC at pH=2. As the pH is below the value of the CMC pKa, most carboxylic groups are protonated, so there is no electrostatic repulsion between the carboxylic groups.

Another interesting observation is the solubility of the CMC gels. The untreated CMC gels, which show little swelling, are clearly more soluble in water. Swelling experiments could not be performed at long times because the gels were dissolved in the external aqueous media. As indicated in Tables 1 and 2, the gels become soluble after certain periods of time. Dissolution was faster at pH=8, and slower at pH=2. This is also due to the ionic character of the polymer at basic pH.

Those results are of interest for application of those gels, in form of microgels in oral delivery over a food product. PH in food products is around pH=6. According to above mentioned results, microgels of CMC would be considered as promising drug delivery vehicles, protecting the active agent at pH=6 and 2 and releasing it at pH=8. After oral delivery of the food product together with the microgel, the product might reaches the stomach, with pH=2, where microgel could remain stable and protect the drug inside. Finally, at pH=8, which is close to the real pH in the intestine, where the nutrients are absorbed and it is interested that the microgel dissolves/swells and releases the active agent there.

Furthermore, there is a clear difference of swelling between the dried CMC and the non-dried. Certainly, the re-hydration of the dried polymer results in an increase of weight, which makes difficult the measure the swelling simply by weight.

6.7. FORMATION AND PROPERTIES OF CMC BEADS AND CAPSULES

As mention before (section 6.5.1) capsules and beads could be obtained by adding $FeCl_3$ to CMC gels. The procedure was very similar to which described in Figure 20, but a CMC gel was used instead of an emulsion (Figure 23). Initial CMC concentration was kept constant at 3 wt%.

Capsules (with a liquid inside) were obtained by quickly removing the particles from the $FeCl_3$ solution. If the gels were left for longer periods of time (30 min for 1wt% $FeCl_3$), then solid beads (solid-like inside) were obtained instead of capsules. Probably, there was an excess of iron ions, which could diffuse inside the particles. Consequently, the cross-linking of carboxylate groups with Fe^{3+} took place in the interface, forming capsules, and continued towards to the interior of the particle. The interesting point is that the thickness of the capsules could be controlled by time.

An example of beads, obtained from CMC gels and with 3wt% CMC and introduced into 1wt% Fe^{3+}, is shown in Figure 23. The orange colour is attributed to the presence of iron salt.

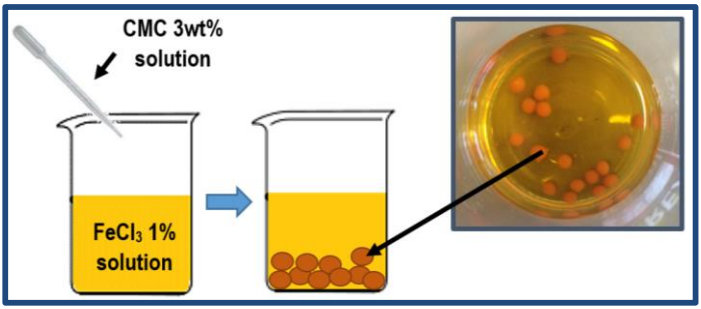


Figure 23. Beads of CMC cross-linked with Fe^{3+} ions

The response of theses beads to pH was studied. These beads were introduced into vials with aqueous solutions of HCl (pH=1), NaOH (pH=13) and milli-Q purified water (pH≈7).

With purified water, it was not observed any change on the beads. It remained insoluble into the solution, without undergoing any change on its structure or swelling. In the vial with HCl, the beads swelled slightly and the colour changed from the initial orange until becoming a colourless swelled bead. This change of colour may be due to that, at acidic pH, the carboxylic groups were protonated, thus neutral, they have lower interactions with the cation Fe^{3+}, in

contrast to their deprotonated form at pH>pKa. Therefore, at acidic pH an ionic exchange occurs, in which Fe^{3+} is substituted by H^+, considering that the concentration of protons is much higher. In any case, Fe^{3+} ions get released from the gel and consequently, cross-linked structure might be lost. This hypothesis seems to be correct, since the colour is released, and simultaneously, swelling is observed. However, the swelling degree was small, and it could not be measured.

In the presence of NaOH, beads swelled as well as in the vial of HCl, and they did not change its colour from the initial orange until being a colourless swelled bead. It maintained its colour since the carboxylic groups were still charged and interacting with the cation Fe^{3+}. The ionic exchange could not be possible, since the cation present (Na^+) has a lower affinity for carboxylate groups.

The colourless bead inside the vial with the HCl solution, in which it lost the cross-linked structure because it lost the crosslinker inside, was introduced after into the vial with NaOH and it dissolved (Figure 24), corroborating the theory of being a drug delivery vehicle.

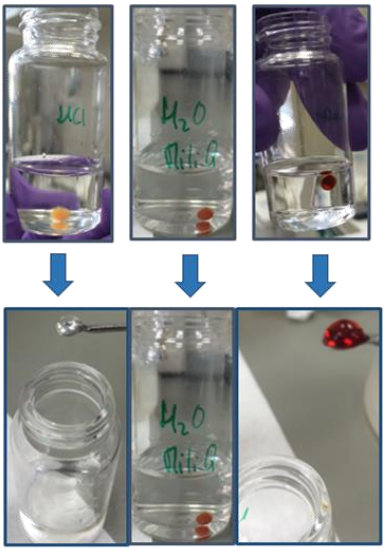


Figure 24. Picture of the behaviour of a bead of CMC cross-linked with Fe^{3+} ions. At acid pH the bead loose the orange colour and swells (left); at neutral pH the bead remains stable without swelling (middle) and at basic pH it does not change its colour and swells.

7. CONCLUSIONS

The main objective of this study consisted in the obtaining and study of biocompatible microgels as a drug delivery system, which could encapsulate an active ingredient to be delivered into the intestine. The results obtained have led to the following conclusions:

1. CMC gel particles remain stable at acid pH but dissolve at basic pH, as it would be in the human body, where acid pH would represent the stomach and the basic the intestine. In this way, the active ingredient inside the CMC microgels would remain stable at acid pH and then it would be released into the intestine, as expected.
2. After having studied the phase behaviour of CMC and BSA mixtures, the results have shown a clear phase separation in to immiscible aqueous phases. In these systems, water-in-water emulsions were obtained. These emulsions remained stable during several weeks, thanks to the addition of small amounts of HCl.
3. Capsules and beads have been obtained, by cross-linking CMC with Fe^{3+}. The thickness of the shell depended on the duration time of the contact between CMC and Fe^{3+}.

The results are interesting, and formation of microgels and capsules will be studied in more detail in further work.

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9. ACRONYMS

CMC – Carboxymethylcellulose

BSA – Bovine Serum Albumin

W/W (emulsion) – Water-in-water (emulsion)

W/O (emulsion) – Water-in-oil (emulsion)

O/W (emulsion) – Oil-in-water (emulsion)

O/O (emulsion) – Oil-in-oil (emulsion)

W/O/W (emulsion) – Water-in-oil-in-water (emulsion)

O/W/O (emulsion) – Oil-in-water-in-oil (emulsion)

PEG – Polyethylene glycol

CGNA – Centro de Genómica Nutricional Agroacuíco

MW – Molecular weight

rpm – Revolutions per minut

pI – Isoelectric point

wt% - percentage by weight