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Grau de Química

Treball Final de Grau

Preparation and characterization of gelatin microgels by calcium carbonate template.

Preparació i caracterització de microgels de gelatina a partir d'una plantilla de carbonat de calci.

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Todo el mundo trata de realizar algo grande, sin darse cuenta de que la vida se compone de cosas pequeñas.

Frank A. Clark

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 gran grup de recerca i deixar-me dur a terme la realització del meu treball final de grau. També m'agradaria donar les gràcies a la Dra. Maria Sarret Pons per implicar-se tant i donar-me la oportunitat de veure el dinamisme del món empresarial en el major organisme públic d'investigació d'Espanya.

Cal destacar que la Dra. Susana Vilchez Maldonado m'ha obert un nou món d'aprenentatge. A despertat un petit buit de curiositat que juntament amb la seva paciència i dedicació, i la de tots els membres de l'equip del centre de química col·loïdal i interfacial, s'ha anat omplint de cada cop més coneixements i, per tant, més dubtes. Aquest equip que m'ha acollit i m'ho ha posat tant fàcil està format per dos grups liderats pel Dr. Jordi Esquena Moret i la Dra. Conxita Solans Marsà i no puc deixar de nombrar alguns dels seus integrants que més m'han ajudat: Dr. Jonatan Miras Hernández, Elena Gamarra Gascó, Albert González Lladó, Rodrigo Magana Rodríguez, Yoran Beldengrün, Dra. Marta Monge Azemar, Dr. Jérémie Nestor, Dra. Maria Homs que han fet d'aquest projecte una experiència realment agradable i molt enriquidora.

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

REPORT

CONTENTS

1. SUMMARY

The following work was focused in the study, preparation and characterization of gelatin microgels based in the structure of a porous $CaCO_3$ template in its polymorphs form vaterite (spherical). The importance of this research is because the template is cheap, non-toxic and easy to produce and to decompose by EDTA at basic pH.

The template was prepared by mixing the same volume of salts, Na_2CO_3 and $CaCl_2$. It was characterized by optical microscopy, scanning electron microscopy (SEM), nitrogen sorption, Thermogravimetric Analysis (TGA) and zeta potential. The obtained results demonstrate that the template has a particle size of about 4 μm with a wide distribution of mesopores.

The microgel has been obtained by two ways by using the $CaCO_3$ template. The first one is the use of physisorption (physical adsorption) and the second one is the co-precipitation. Only the second way provide quantitative results.

As a gelatin cross-linker, genipin and transglutaminase have been chosen by its nature, biocompatibility and low toxicity. Genipin has given the best results because transglutaminase could be more influenced by pH and may contain partial charges.

Finally, gelatin microgels have been characterized by SEM, optical microscopy, TGA and Z potential. Microgels cross-linked with genipin have a size distribution similar to $CaCO_3$ microparticles and a slight negative charge in solution.

Keywords: microgels, calcium carbonate, gelatin, co-precipitaton, physiosorption, genipin, transglutaminase

2. RESUM

L'objectiu d'aquest treball és l'estudi, l'obtenció i la caracterització de microgels de gelatina mitjançant la utilització d'una plantilla porosa de $CaCO_3$ en la seva poliforma vaterita (esfèrica). Aquesta recerca té la seva importància per la no toxicitat, assequible i fàcil de produir i per la facilitat de descomposar la plantilla amb EDTA a pH alcalins.

La plantilla ha sigut preparada barrejant el mateix volum de Na_2CO_3 i $CaCl_2$. S'ha caracteritzat mitjançant microscòpia òptica, microscòpia electrònica de rastreig (SEM), sorció de nitrogen, anàlisi termogravimètrica (TGA) i potencial zeta. Els resultats demostren que s'obté una plantilla mesoporosa amb una ampla distribució de mides amb un tamany d'unes 4 μm.

En la obtenció del microgel s'han utilitzat dues vies partint de la plantilla de $CaCO_3$. La primera consisteix en l'aprofitament de la fisisorció (adsorció la segona en la co-precipitació. Només de la segona via s'han pogut obtenir resultats quantitatius.

Com a reticulants de la gelatina, s'han escollit la genipina i la transglutaminasa per la seva naturalesa, biocompatibilitat i baixa toxicitat. La genipina és la que ha donat millors resultats ja que a la transglutaminasa li afecta molt més el pH i pot contenir cargues parcials.

Finalment, s'han caracteritzat els microgels mitjançant SEM, microscòpia òptica, TGA i potencial Z. Els microgels reticulats amb genipina han proporcionat una distribució de mida similar a les micropartícules de $CaCO_3$ i una petita càrrega negativa en solució.

Paraules clau: microgels, carbonat de calci, gelatina, co-precipitació, fisisorció, genipina, transglutaminasa.

3. INTRODUCTION

Multicomponent system can be classified by their particles size:

- Mixtures and solutions: with a particle size smaller than 1nm.
- Colloidal systems: with a particle size between 1nm-1μm.
- Suspensions: with a particle size bigger than 1μm.

Which is important to know for this project are the colloidal systems. A colloidal dispersion is a system where the disperse phase (more-or-less compact particles) is dispersed in the continuous phase (macroscopic) in a non-equilibrium situation. One example of a colloidal system are gels.

3.1. GELS

A gel is a nonfluid colloidal polymer network, which is swollen by a suitable solvent. It is named hydrogel if the solvent is water and organogel if the solvent is organic. A gel is a three-dimensional structure and its stability is determined by physical or chemical cross-links of the network [1]. A cross-linker is a substance that creates a bond which links one polymer chain (synthetic or natural) to another.

In a physical cross-linked gel, the interactions between polymers are established by ionic, hydrogen bonding, electrostatic and/or hydrophobic interactions, or crystallization. It has the advantages of reversibility and absence of chemical reactions potentially harmful to the integrity of incorporated bioactive agents or cells [2].

In a chemical cross-linked gel, covalent bonds are formed between polymer chains. It allows the formation of gels with controllable mechanical strength and superior physiological conditions stability [3].

3.1.1. Microgels

Microgels are colloidal particles having an internal gel structure. Hence, it is a 'soft' cross-linked polymer network that form particles with diameter in the range of hundreds of nanometers to several micrometers[1]. These polymers can be naturals or synthetics. Naturally derived polymers including proteins and polysaccharides have advantages of biocompatibility, biodegradability, and ease of chemical modification in relation to synthetic polymers [4].

Nano- or microgels can be prepared by heterophase polymerization with different cross-linkers in aqueous phase[5]. The possible processes include precipitation polymerization, water-in-oil emulsion polymerization[6], and micro-emulsion polymerization[7]. Among these, precipitation polymerization is probably the most frequently used technique.

Microgel manufacture techniques can be classified by their methods routes:

- Physical: involves gelling the droplet phase of an emulsion or spray. It is not a manufacturing method in its own right, but it is the precursor for many of chemical methods.

- Physicochemical: has two distinct paths -coacervation (attraction between mixed polymers) and phase separation (repulsion between mixed polymers)[2].

There are other classifications by looking at:

- its precursor- monomer, polymer or macro-gel.

- physical formation of precursor droplets by emulsion, atomisation or microparticulation route[8-11].

To get the microgel desired shape it can be used emulsion way or a template. There are various types like anionic sulfonated polystyrene (PSS) particles, dicationic poly(4-vinyl pyridinium) ionic liquid capsules, porous TiO_2 materials, but which is of interest by their biocompatibility, biodegradability, non-toxicity and because is cheap and easy to produce and to decompose is a $CaCO_3$ template[12]. If emulsion way would have been selected, template is not necessary.

3.2. $CaCO_3$ MICROPARTICLES

The calcium carbonate microparticles can have three polymorph forms according to the conditions of synthesis: aragonite (star), vaterite (sphere) and calcite (diamond) as it is showed

in Figure 3.1. It is obtained from direct mixing of salts Na_2CO_3 and $CaCl_2$[13]. The initial precipitate is amorphous and eventually transforms into aggregated $CaCO_3$ microcrystals with a particular morphology. This route achieves uniform, homogeneously sized and highly porous spheres. The quality of the resultant microparticles is strongly dependent on the experimental conditions.

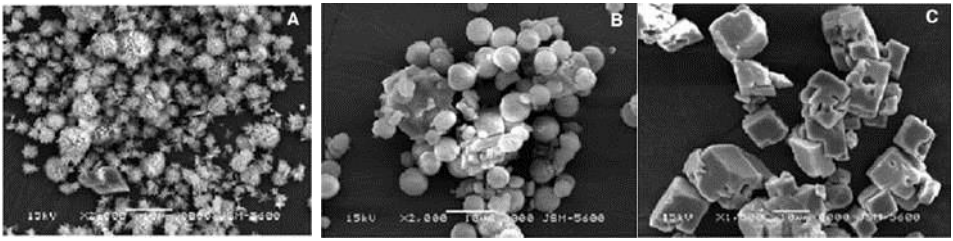

Figure 3.1:Scanning electron micrographs of $CaCO_3$ polymorphs:(A)aragonite (B)vaterite (C)calcite[14].

To be used as a template the most suitable form is vaterite; despite being the most unstable form, its spherical and nanoscale porous structure is the most convenient. A limitation is that vaterite recrystallizes in presence of water and humidity to non-porous calcite, the most stable form, within some hours.

Temperature during crystallization has a strong effect on the polymorphs formation. The optimal temperature for vaterite crystal growth is in the range 20-50ºC. Salt concentration also has influence in the size of the crystals. At higher concentrations smaller crystals are obtained and inversely, at lower concentrations, bigger are the crystals. If mixing conditions are controlled (time and stirring) it is possible to prepare monodispersed $CaCO_3$ without any additives [14, 15].

Using these template two main routes to load $CaCO_3$ cores with polymers have been elaborated: physiosorption and co-precipitation [14].

3.2.1. Physiosorption

This method takes advantage of the fact that the template is formed by porous channel-like voids that can be filled with the material of interest. It consists of filling the pores of synthesized $CaCO_3$ cores by physically adsorbed protein. It is based on unspecific molecular interaction on the pore surface. Protein adsorption is strongly driven by electrostatic interactions [14].

3.2.2. Co-precipitation

It is based on a capture of protein during the core preparation but the exact mechanism is still unknown. It is not clear how the proteins may affect the internal structure of the cores. In the process, a better yield has been obtained by co-precipitation than by physical adsorption. Nowadays co-precipitation is the most popular method because its high efficiency in spite of the possibility that those small molecules can easily diffuse out from the interconnected pores. This process is easiest with doped charged macromolecules, due to physical interaction between them [14].

3.3. GELATIN

Gelatin is a protein obtained by the partial hydrolysis of collagen which is getting from the skin, from connective tissue and bones of animals. When its hydrolysis is derived from an acid-treated it is known as Type A and when it is derived by an alkali-treated process it is called Type B [16-19].

Collagen is the major insoluble fibrous protein in the extracellular matrix and the most abundant protein in the animal kingdom. It contains an unusually high level of cyclic amino acids proline and hydroxyproline. Collagen consists of three helical polypeptide chains wound around each other and connected by intermolecular cross-links.

In gelatin, the formation of thermoreversible gels is one of the most important properties and it is indicated by Bloom number. It measures gel strength for a known concentration in a heterogeneous mixture of water-soluble proteins. A gelatin with a 225g Bloom corresponds to a high average molecular weight between 50-10 KDa presents in collagen [20].

The amount of triple helices in gelatin hydrogels depends on gelatin concentration, thermal treatment and aminoacid composition. The presence of sugars and other polyols enhances the formation of triple helices due to carbohydrates bind water in their hydration shells enhancing protein-protein interaction and thus helix formation [21].

Gelation of gelatin also involves stabilising hydrogen bonds to form the triple helical structure although gelatin does not undergo the aggregation step. It has emerged like a hydrogel with soft material and tuneable properties and has been found that has widespread use in various applications[22].

Cross-linking is one of the most important procedures for modification polymer networks [1]. If a cross-linker is used, interactions between the polymer chains are enforced and the polymer adopts a fixed conformation that hinders to be disrupted with environmental conditions. The cross-linker type and its content can influence many properties of microgels and microparticles [4]. Among them, genipin and microbial transglutaminase are good options for its low-toxicity, biodegradability, biocompatibility and non-immunogenicity.

3.4. GENIPIN

Genipin is a natural crosslinker that comes from gardenia seeds and has low cytotoxicity [23, 24]. It 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 disavantatge of genipin is that the crosslinking reaction results in a dark blue coloured gel which limits use in in several applications [2]. One of the most accepted mechanisms proposed for the reaction between gelatin and genipin is in Figure 3.2.

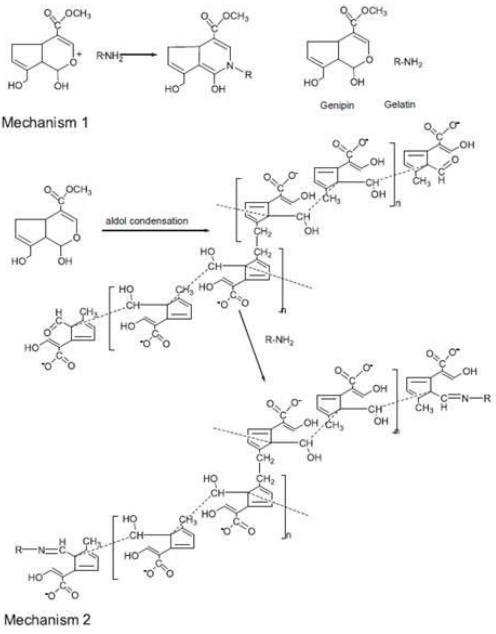

Figure 3.2: Proposed mecanism for gelatin-genipin cross-linking[25].

3.5. MICROBIAL TRANSGLUTAMINASE

Transglutaminase is a thiol-enzyme which acts as a catalyst in a reaction between the c-carbonyl group of glutamine residue and the e-amino group of a lysine residue inducing covalents bonds and isopeptide bond formation. There are two types, microbial (mTGAse) and mammalin tissue (tTGAse). Although tTGAse has a higher reactivity than mTGAse, it has the disadvantage that requires calcium to be present for its activity. The process and the concentration of TGAse affect the cross-link stability over time and if it is insufficient it goes to a thermal physical gelation in a weaker gel forms. Gelatin crosslinked with TGAse is stable from 37ºC and has the potential to allow microgels to be produce with a variety of functionalities [2, 26].

3.6. APPLICATIONS OF MICROGELS

Gelatin has been widely used as biocompatible material for medical, pharmacy, cosmetic, textiles and food products, and moreover new gelatin-based materials are being developed [27] because of its many advantageous properties, such as low-toxicity, biodegradability, biocompatibility and non-immunogenicity.

Particularity, gelatin nano and microgels have attracted much attention in tissue engineering and drug delivery applications in recent years and different methods have been developed such as micromolding methods, microfluidic preparation, spray drying, freeze drying, emulsification among others, but these processes required organic solvents, surfactants and/or expensive processing equipments. That is why it is urgently needed to develop an environmentally friendly method [13].

If what is sought is the concrete application in food and in textile scope it must be taken into account that the cross-linker meets the requirements of low-toxicity, biodegradability, biocompatibility and non-immunogenicity too. Transglutaminase and genipin fullfill these objectives with their potentially and that is why it has been chosen [2].

4. OBJECTIVES

Giving the importance of finding an ecofriendly, biocompatible and non-immunogenic route to obtain gelatin microgels and, taking into account the considerations explained in the introduction, the main objective of this work was to obtain gelatin microgels by a calcium carbonate template and to characterize them.

To make this project possible, it was divided into subsections:

- Synthesis of CaCO3 template in vaterite polymorphism. Characterization of microparticles by opical microscopy, scanning electron microscopy (SEM), nitrogen sorption, thermogravimetric analysis (TGA) and zeta potential.
- Preparation of gelatin microgels by physiosorption and co-precipitation by using $CaCO_3$ template and two different cross-linkers, genipin and transglutaminase.
- Characterization of gelatin microgels by optical microscopy, scanning electron microscopy (SEM), thermogravimetric analysis (TGA) and zeta potential.

5. EXPERIMENTAL SECTION

5.1. MATERIALS

- **Sodium carbonate anhydrous**: Na_2CO_3, with a molecular weight of 105.99 g/mol. Supplied by Panreac Química S.A.
- **Calcium chloride:** $CaCl_2$, 93 wt. %, with a molecular weight of 110.98 g/mol. Supplied by Sigma-Aldrich.
- **Gelatin:** from bovine skin, 225g Bloom. Supplied by Sigma-Aldrich.
- **Fluorescein 5(6)-isothiocyanate (F):** $C_{21}H_{11}NO_5S$ BioReagent, suitable for fluorescence, mixture of 2 components, ≥90% (HPLC). With a molecular weight of 389.38 g/mol. Supplied by Sigma-Aldrich. (Figure 5.1)
- **Dimethyl sulfoxide (DMSO):** $(CH_3)_2SO$ ACS reagent, ≥99%, with a molecular weight of 78.13 g/mol. Supplied by Sigma-Aldrich.
- **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. (Figure 5.2)
- **Microbial transglutaminase (mTGAsa)** used as a crosslinker**:** Ajinomoto Transglutaminase Preparation. Supplied by Activa ® WM.
- **Phosphate buffered saline (PBS),** in tablets: It provides a pH = 7.4 (physiological pH) at 25 ºC. It consists of a mixture of $Na_2HPO_4 \cdot 2H_2O$ (11.88 g/L) and KH_2PO_4 (9.08 g/L). Supplied by Sigma-Aldrich.
- **Ethylenediaminetetraacetic acid (EDTA):** $C_{10}H_{16}N_2O_8$, BioUltra, anhydrous, ≥ 99% (titration). With a molecular weight of 292.24 g/mol. Supplied by Sigma-Aldrich.
- **Di-phosphorus penta-oxide**: P_2O_5, 98% wt.%, with a molecular weight of 141.94 g/mol. Supplied by Panreac.
- **Filtered deionized water:** H_2O, Milli-Q® water (ultra-pure Millipore water system, Milli-Qplus 185 filter). Supplied by Merck Millipore.

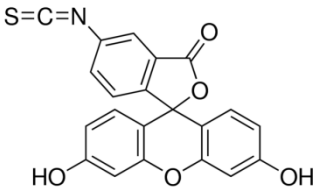

Figure 5.1: molecule fluorescein 5(6)-isothiocyanate

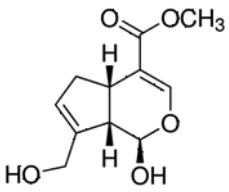

Figure 5.2: molecule genipin

(13/12/16 by Sigma-Alcrich catalog)

5.2. APPARATUS AND INSTRUMENTAL

The equipment and instruments are listed below:

- **Analytical balance** Mettler Toledo PM200 with a precision of $\pm 10^{-3}$g (maximum capacity: 200g).
- **Basics magnetic stirrer hot plate** Heidolph MR Hei-Standard with temperature controlled by means of Heidolph EKT3001 probe (maximum stirring capacity: 2500 rpm).
- **Basics magnetic stirrer hot plate** IKA® RCT. (maximum stirring capacity: 1500 rpm).
- **Vortex shaker** IKA® VORTEX 3 GENIUS 3.
- **Vortex shaker** Heidolph Reax Top.
- **pH-meter** Mettler Toledo Seven Easy.
- **Oven** KOTTERMANN 2712 (maximum temperature: 250 ºC).
- **Lyophiliser** Christ Alpha 2-4 LD Plus, with pressure and temperature of ~0.03 mbar and -85 ºC, respectively.
- **Tabletop Scanning Electron Microscope**(SEM) Hitachi TM-1000
- **Centrifuge** Eppendorf 5804R
- **Optical microscope** Olympus BX51TRF-6
- **ZetaSizer** Nano-Z Malvern Instruments
- **Automated gas sorption analyzer** Autosorb iQ Quantachrome

5.3. PREPARATION OF $CaCO_3$ MICROPARTICLES

Vaterite $CaCO_3$ microspheres were prepared rapidly mixing equal volumes of Na_2CO_3 0.33M and $CaCl_2$ 0.33M for 30 seconds at 1200 rpm and 30ºC [13]. The resultant $CaCO_3$ microspheres were extracted by Buchner funnel, washed three times with deionized water and dried at 65ºC in an oven.

It is very important to maintain these particles in a desiccator with P_2O_5 to avoid the contact with the air and prevent its crystallization to its stable form, calcite.

5.4. GELATIN MICROGEL

Two main routes to load gelatin to the $CaCO_3$ cores have been elaborated.

5.4.1. Preparation of microgel by physiosorption

Physical adsorption is based on unspecific molecular interaction on the pore surface of $CaCO_3$ cores [13].

It consists of mixing equal volumes of 6% or 4% $CaCO_3$ redispersed microparticles and 15% gelatin for 6 hours at 750 rpm, 50ºC. Then two procedures have been done. The first one consists of three centrifugations, water rinsing, redispersions cycles at 1000 rpm, 25ºC for 5 minutes. In the second one, the centrifugation was replaced by sedimentation for 48 hours.

Once we have the microgel, the next step consists of cross-linking gelatin. The microgel was redispersed and filtered with the cross-linker in equal volumes and let it react for 16 hours at 30ºC. Two types of cross-linkers have been used. The first one was genipin 0.028M. It always has been prepared in situ and shaken for 1 hour at room temperature, at 750 rpm with the aim of always having the same experimental conditions because genipin polymerized with time [23, 24]. The other one was microbial transglutaminase (mTGAse) that was prepared at 6%. The only difference between both cross-linkers is that mTGAse has to be deactivated heating it for 5 minutes at 70ºC.

After cross-linking it is necessary to perform centrifugation, water rinsing, redispersions cycles at 1000 rpm, 25ºC for 5 minutes by eliminate non adsorbed gelatin.

Then the treatment with EDTA 0.1M at neutral pH to remove the $CaCO_3$ template has been done. An excess of EDTA was poured into the microgel redispersion and it was shaken at 500 rpm for 30 minutes. After that, two centrifugations cycles at 3000 rpm and at 25ºC for 5 minutes was carried out. The supernatant was removed and it was rinsed with EDTA after each centrifugation. Finally, three centrifugations cycles at 1000 rpm and at 25ºC for 5 minutes was performed by rinsing with MilliQ water after each cycle.

The last step was to freeze with dry-ice and acetone, lyophilize and characterize the final product.

5.4.2. Preparation of microgel by co-precipitation

Co-precipitation method allows introducing protein into the $CaCO_3$ particles with high amount and using a single step [14].

It consists of mixing equal volums of 15% gelatin and $CaCl_2$ 0.33M at 750 rpm until it was homogeneous. Then Na_2CO_3 0.33M is poured into the mixture and blended at 1200 rpm, 30ºC for 30 seconds. The solution is let to react for 15 minutes and after that, centrifuged three times at 3000 rpm, 25ºC for 2 minutes. The sample, after each centrifugation cycle, was rinsed and redispersed with MilliQ water.

Once the microgel has been cleaned the same way as in physisorption method has been followed. The next step consists of cross-linking gelatin. The microgel was redispersed with the cross-linker in equal volumes and let to react for 16 hours at 30ºC. Two types of cross-linkers have been used, genipin 0.028M and mTGAse 6%. The process has followed the same way and conditions as in section 5.4.1.

After crosslinking it was necessary to remove non-adsorbed gelatin by performing two centrifugations cycles at 2000 rpm and 25ºC for 2 minutes. The sample was rinsed and redispersed in MilliQ water after each centrifugation. The following step has been done by two different ways. The first one consisted on filtering by number 3 filter and the second one, replacing the first one, consisted on transferring our mixture into a Petri dish and placed it in an oven for 3 hours at 40ºC, to evaporate the solvent.

Then the treatment with EDTA 0.1M at pH=11 to remove the $CaCO_3$ template has been done. It consists in poured excess of EDTA in the microgel redispersion and shaken it at 500 rpm for 2 hours. After that, the supernatant was eliminated with two centrifugations at 2000 rpm, 25ºC for 2 minutes, using EDTA rinsings and redispersions cycles. And finally, two more centrifugations were performed at the same conditions but in this occasion MilliQ water rinsing, redispersions cycles were used with the objective of get a clean sample. To avoid the aggregations, the solution is ultrasonicated for 15 minutes.

With the aim to confirm that gelatin was present after each step, the initial gelatin was labelled with different concentration of fluorescein (0.5, 1 and 2%) dissolved in dimethyl sulfoxide (DMSO). So, various performing gelatin labelled has been done to choose the best one percentage.

The last step was frozen with dry-ice and acetone, lyophilized and characterized the final product.

5.5. CHARACTERIZATION

5.5.1. Optical Microscopy

There are various techniques in Optical Microscopy: bright field, dark field, polaritzed light, phase contrast, differential interference contrast (DIC) and fluorescence among other. In $CaCO_3$

characterization bright field and polarized light has been used. In microgels characterization the most used has been DIC and fluorescence [28, 29].

5.5.1.1. Bright field

Sample illumination is obtained throw a beam light from below and observed for above. The contrast with white light is getting by absorbance which is who allows us to see the image. Their biggest advantage is its simplicity, although its main limitations are that it is obtain very low contrast and low apparent optical resolution [30].

5.5.1.2. Polarized light

This technique is based in the influence of a polarized beam on a second polarized sheet. The light comes from a standard lighting source and vibrates and spreads in all directions but when it goes through a polarized filter, the waves and the electric field oscillate in the same plane. Electromagnetic waves vibrate in a perpendicular direction to the displacement. Depending on the angle between the two sheets, it will obtain a certain intensity and wavelength. If the two blades are parallel, all the light will be transmitted but if they are perpendicular, the beam will not be able to cross the second.

The aim of this technique is to obtain close information about sample structures using the advantage that some of them may be capable of changing the polarization plane of light. The anisotropic materials have different refractive index in the direction of the light beam, whereas the isotropic materials have a constant index of refraction.

5.5.1.3. Differential interference contrast

This technique is capable to give us information about surface and internal structure of materials. It uses polarized light which passes through two birefringent prisms that divide it into two rays whose paths and polarization axes are different and then are passed through the prism of Wollaston. As a result there is an optical path difference. For the interference of both, the vibrations of the beams of different path length must be carried to the same plane and axis. To do this, a second polarizer/analyser is placed next to the recombinant Wollaston prism. The light beam passes through the analyser that gets them to the same plane and axis. Thus an interferential difference occurs between the two originally independent beams and that it finally makes possible observe a 3D sample with differences in intensity and colour. These differences depend on the refractive index and/or thickness of the sample.

5.5.1.4. Fluorescence

The sample is illuminated with light of a specific wavelength that corresponds to its properties, (fluorescein emit at 495 nm) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (a different colour than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The microscope used for the microgel characterization uses an halogen lamp as a light source that emits from 400 nm, an excitation filter, a dichroic beam splitter and an emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the sample. All of this is possible because gelatin has been labelled with fluorescein, a green fluorescent, amine-reactive label [31, 32].

5.5.2. Scanning Electron Microscopy

In this technique an electron beam is accelerated and used to illuminate a sample that must be emptied. These electrons lose energy due to different mechanisms of interaction. The electrons that lose that energy with the volume of interaction cause the emission of secondary electrons, which are detected by the photomultipliers of the microscope and gives information about the topography and morphology of the material [33, 34].

Hitachi TM-1000 tabletop scanning electron microscope was used to take micrographs and by the Image J software the size distribution of the microgels has been measured. At least more than 500 measurements per sample were performed.

5.5.3 Nitrogen sorption technique

Nitrogen sorption technique allows characterizing porous materials by different parameters such as surface area, pore size, pore volume, etc. These parameters are obtained by applying different theoretical models.

By measuring the amount of physically adsorbed gas according to the Brunauer-Emmett-Teller (BET) method, determination of the specific surface area was obtained. It requires a linear plot of $1/[W(P_0/P)-1]$ vs. P/P_0 which for most solids, using nitrogen as the adsorbate, is restricted to a limited region of the adsorption isotherm. Usually the results only adhere to the

P/P_0 range of 0.05 to 0.35. This linear region is shifted to lower relative pressures for microporous materials whose diameter porous do not exceed 20 Å.

Barrett-Joyner-Halenda theory (BJH) is typically applied on mesoporus materials (diameter microparticles of 20-500Å) [35]. This method determinates mesoporous distribution. In BJH method it is assumed that the initial relative pressure is close to unity and all pores are filled with liquid. When the relative pressure is lowered from $(P/Po)_1$ to $(P/Po)_2$ a volume V_1 will desorb from the surface. V_1 represents not only emptying of the largest pore of its condensate, but also a reduction in the thickness of its physically adsorbed layer. It uses the modified Kelvin equation to relate the amount of adsorbate removed from the pores of the materials, as the relative pressure is decreased from a high to low value, to the size of the pores.

Mesopore size calculations are given by Kelvin equation assuming cylindrical pore geometry showed in Equation 5.1:

$$r_K = \frac{-2\gamma V_m}{RT \ln(P/P_0)}$$

Equation 5.1: Kelvin Equation

γ : the surface tension of nitrogen at its boiling point (8.85×10^{-7} J/cm^2 at 77 K).

Vm = the molar volume of liquid nitrogen (34.7 cm^3/mol).

R = gas constant (8.314 J/Kmol).

T = boiling point of nitrogen (77 K).

P/P_0 = relative pressure of nitrogen.

r_K = the Kelvin radius of the pore in which condensation occurs.

But the actual pore radius r_p is given by: $\boldsymbol{r_p = r_k + t}$ where t is the thickness of the adsorbed layer (Vads/Vm).

The amount of gas adsorbed is a function of:

- The interaction strength between gas and sample (intrinsic).
- Temperature (fixed at 77K).

- Pressure (controlled variable) and expressed as P/P_o.

These techniques are based on an adsorption/desorption process of a gas (N_2) at low temperature and it is represented by an isotherm obtained from analyser software. This isotherm could provide some information as if the degassing has been done correct or more temperature is needed, porous size and distribution, etc.

In this work it is used an Automated gas sorption analyzer Autosorb iQ Quantachrome. The sample was degassed into a cell for 12 hours using a ramp of 1ºC every 5 minutes until it has arrived to 90ºC. Then it was maintained at this temperature for 1 hour. Once the sample is prepared for measurement, the instrument is able to get the expected parameters from BET and BJH theories [14, 36, 37].

5.5.4 Thermogravimetric Analysis

It is a method of thermal analysis that measures the amount of weight change of a material, either as a function of increasing temperature, or isothermally as a function of time, in an atmosphere of nitrogen, helium, air, other gas or in a vacuum.

Thermogravimetric analysis (TGA) is used as one of several complementary techniques in the identification of an unknown polymer composite to find the weight percent of each material. The TGA curve shows the different temperatures of combustion or evaporation [38].

The sample was weighted in a microbalance Mettler Toledo XPR2, placed in an alumina crucible and put it in a thermobalance Mettler Toledo TGA/SDTA 851e. Then, it was heated from room temperature to 900ºC at a rate of 10ºC/min in nitrogen atmosphere. The curve result was obtained by the percentage of weight vs. increasing temperature [39].

5.5.5 Zeta potential

The zeta potential (ζ) is the electrical potential between the dispersion medium and the stationary layer of the fluid attached to the particle dispersed at the hydrodynamic plane of shear and it depends on the distance of this particle as it can be seen in Figure 5.3.

When a charged particle is suspended in a solution, the opposite charge ions will be attracted to the surface of this particle. While the ions closest to the surface of the particle will

be strongly attached to it, the more distant ions will bond more weakly to and this is what is known as the diffuse layer. In that layer, there is a hypothetical limit where any ion within that limit will move with the particle when it moves inside the liquid, but any ion outside that limit will stay where it was originally. This limit is known as a slipping plane.

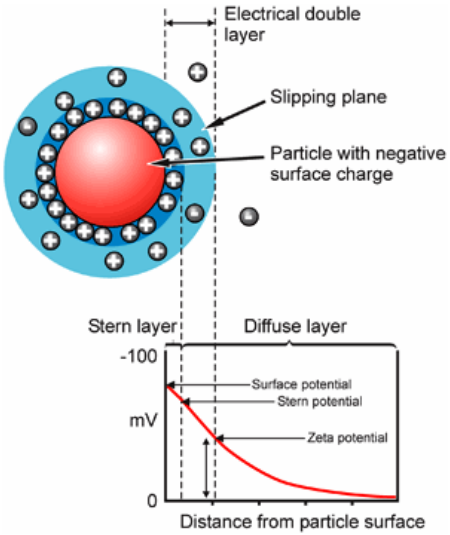

Figure 5.3: Theoretical model for Z potential definition

(13/12/16 by www.scai.uma.es/servicio/aqcm/eel/eel.html)

The most common method to determine Z potential is through the measurement of the speed of motion of a particle in a liquid when an electric field is applied. So, what really measures zetasizer is the electrophoretic mobility of the particles and through the application of Henry equation (Equation 5.4) calculates Z potential.

This equation depends on the activity/concentration of the sample. It has been chosen 1.656 refractive index for $CaCO_3$ microparticles and 1.350 refractive index for gelatin microgel

because ZetaSizer Nano-Z Malvern Instruments is equipped with a laser of 632.8 nm wavelength[39, 40].

$$\mu elf = \frac{velf}{E} = \frac{2\varepsilon\zeta}{3\eta} f(ka)$$

Equation 5.4: Henry equation

μ_{elf}: electrophoretic mobility v_{elf}: electrophoretic velocity

E: electric field ζ: Z potential

η: refractive index ε: relative permittivity

a: particle radius k: Debye length

f(ka): Henry's function=1.5

As it can be seen in equation 5.4, ζ value is influenced by the dispersant so, pH and the ionic strength of the medium are very important.

This method combines the Smoluchowski equation with the measured slope of current–time relationship in electroosmotic flow. Henry's function are generally used as approximations for de f(Ka) determination but because electrophoretic measure was made in a aqueous media and moderate electrolyte concentration, Smoluchowski approximation was applied giving it a value of 1.5.

In addition, Z potential depends on the surface charge of the particle and then, it gives information about the stability of the system. If the particles have low zeta potential values then there is no force to prevent the particles coming together and aggregating. The general dividing line between stable and unstable suspensions is generally taken at either +30mV or -30mV. Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable [40-43].

6. RESULTS AND DISCUSSION

6.1. PREPARATION OF $CaCO_3$ MICROPARTICLES AND CHARACTERIZATION

To be used as a template, $CaCO_3$ microparticles must be obtained with a high yield, with a correct size distribution (low polydispersity) and with the polymorph form of interest, vaterite. With this objective, the preparation of $CaCO_3$ microparticles was performed using the method previously reported (Experimental 5.3); Table 6.1 summarizes the yield and polymorph obtained using different amounts of salts.

Entry	Volum Na_2CO_3 **0.33M**[mL]	Volum $CaCl_2$ **0.33M** [mL]	yield **obtained** [%]	$CaCO_3$ **polymorphs**	pH
1	10	10	65	vaterite	7.44
2	20	20	61	vaterite	7.47
3	50	50	67	vaterite/calcite	7.89
4	100	100	71	calcite	8.49

Table 6.1: Amounts of salts and results obtained in the $CaCO_3$ preparation.

This results show an increment of pH. This fact could be an indication of lack of washing after microparticles preparation.

Taking advantage that the polymorphs can easily be distinguished by their morphology, SEM micrographs have been obtained at each condition. In Figure 6.1 it is easily appreciated that in (a) and (b) the vast majority of microparticles have the rounded morphology corresponding to the desired polymorph, vaterite. In (d) most of microparticles have crystallized to its stable form calcite, but in (c) it vaterite is present but it can be seen that some particles have begun to crystallize.

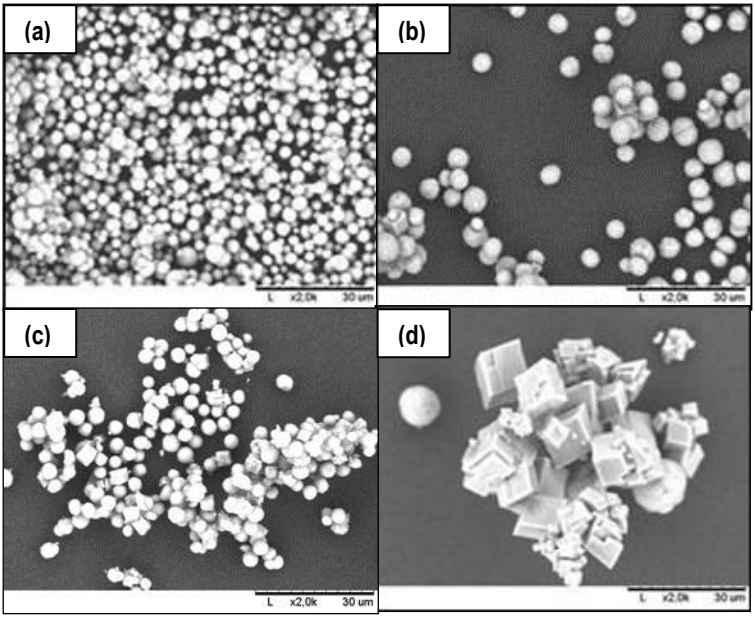

Figure 6.1: SEM micrographs of $CaCO_3$ microparticles[x mL $CaCl_2$+ x mL Na_2CO_3 (a) x=10 (b) x=20 (c) x=50 (d) x=100].

That is why Entry 3 was compared with calcite by Optical Microscopy using bright field and polarized light and the suspicions were confirmed (Figure 6.2): some aggregates close to crystallization were observed. This fact and the increased pH observed in these conditions were the reasons to select conditions of Entry 2 to obtain the $CaCO_3$ microcrystals.

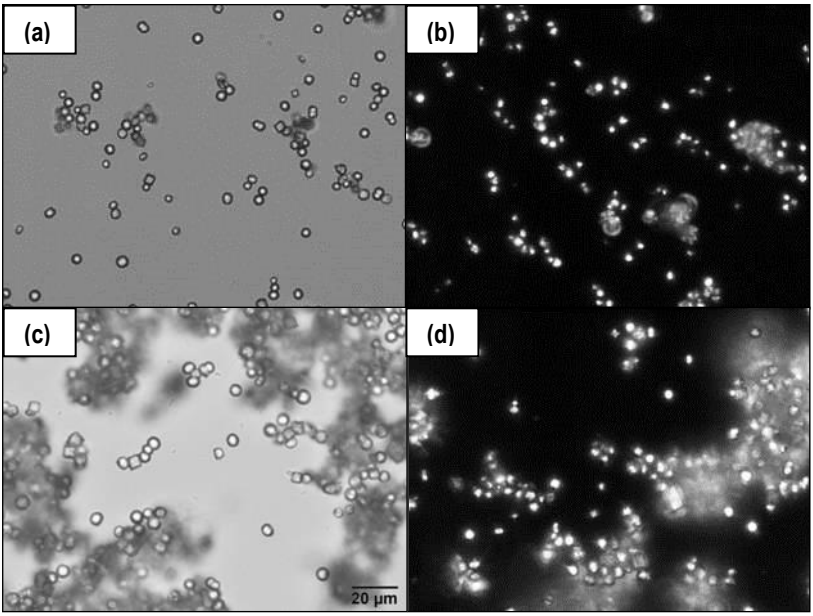

Figure 6.2: Optical microscopy micrographs of $CaCO_3$ microparticles (a) Entry 3 in bright field (b) Entry 3 in polarized light (c) calcite in bright field (d) calcite in polarized light.

Once the preparation method was selected, $CaCO_3$ microparticles were characterised. Their size distribution has been measured from SEM micrographs using the program Image J. More than 500 freshly prepared microparticles were measured. As seen in Figure 6.3, the sample was rather polydisperse, with an average size of 4 ± 2 μm.

	Mean	Standard Deviation	Minimum	Median	Maximum
Particle Size [μm]	4.25	2.24	0.75	4.25	7.75
Relative Freqüency	6.67	5.05	0	7.13	15.65

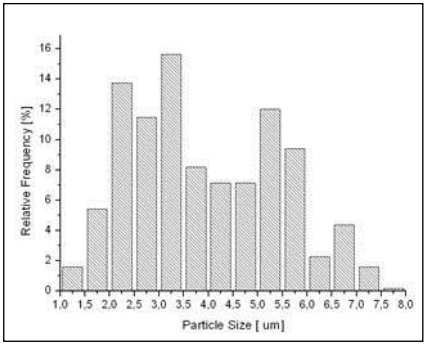

Figure 6.3: $CaCO_3$ μm size distribution.

The surface area and pore distribution were analysed by Nitrogen sorption technique; the experimental isotherm (Figure 6.4) indicates that mild $CaCO_3$ microparticles are a porous material. The sample is probably macroporous because it can be appreciated a hysteresis cycle at high relative pressures, but there are also mesopores, indicated with the hysteresis cycle at intermediate relative pressures. At low relative pressure it is obtained a very low volume and it indicates that there are not micropores (In the case that microparticles would have had micropores, the isotherm would have started at higher volumes).

So, it can be concluded that $CaCO_3$ microparticles are mainly a macroporous material with presence of some mesopores.

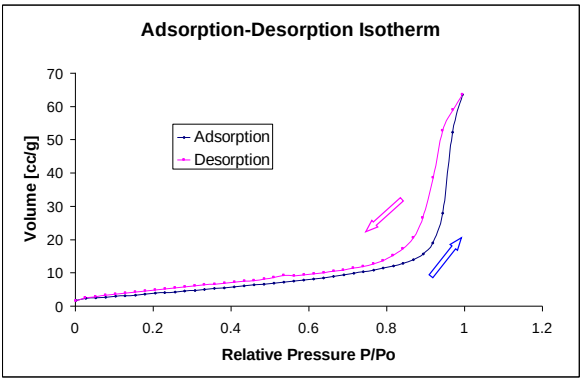

Figure 6.4: Adsorption-Desorption Isotherm in $CaCO_3$ microparticles.

BJH model allows to obtain the pore size distribution by desorption of $CaCO_3$ microparticles. Figure 6.5 shows two maximums. The first one corresponds to the pores in the microparticles with a diameter of 4.28 nm (mesopores) that have a narrow distribution; the second one has a maximum of 22.54 nm (mesopores), but it has a width of more than 50 nm. That could correspond to the adsorption the interstice between microparticles, but also indicates that the sample also contains macropores. It must take into account that automated gas sorption analyzer used do not get pore diameter measures higher than 363 nm. In order to know macropores distribution it would be necessary to use another instrument capable to increase relative pressure and use liquid Hg instead of N_2.

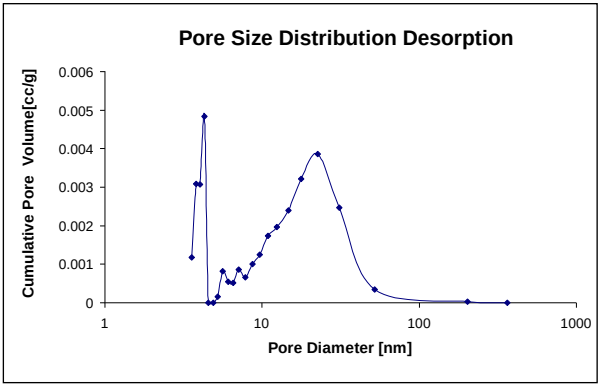

Figure 6.5: Pore Size Distribution by Desorption of $CaCO_3$ microparticles.

Surface Area by BET method, calculated between 0.05-0.30 relative pressure, gave an experimental value of 15.63 m^2/g with a mean diameter of 4.25 μm, a closer result to 8.80 m^2/g with a mean diameter of 5 μm [44] and not so close but with the same magnitude order 40.02 m^2/g with a mean diameter of 5 μm too [39].

By BJH method, a pore volume of $9.8x10^{-2}$ cc/g and a pore diameter of 4.28 nm were obtained.

Since the objective is to use $CaCO_3$ as a template, it will be eliminated with EDTA once the microgel was obtained. To check if the elimination is complete, a thermogravimetric analysis will be performed. Then, a blank of $CaCO_3$ was previously analyzed (Figure 6.6). This technique allows us to know that $CaCO_3$ discompose in a range of 600-700 ºC.

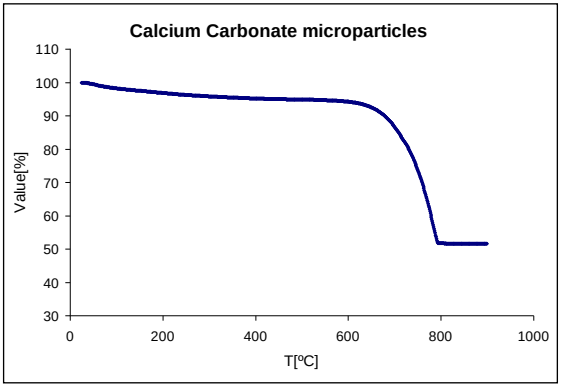

Figure 6.6: TGA $CaCO_3$ microparticles

Finally, in order to know the surface charge and the stability of $CaCO_3$ microparticles, the zeta potential was determined by measuring six replicates.

From measurements, the electrophoretic mobility of 1.57 μcm/Vs was obtained and by the Smoluchowski approximation a zeta potential of -15 ± 6 mV was calculated. This low value indicates that $CaCO_3$ microparticles have a slightly negative surface charge, not enough to maintain the particles dispersed. It was observed that microparticles immediately tended to aggregation and to coagulated after 24 hours.

6.2. PREPARATION OF MICROGELS BY PHYSIOSORPTION

Once the template has been synthesized and characterized, gelatin microgels have been prepared by the physiosorption method. Two different percentages of $CaCO_3$ microparticles and two types of cross-linkers have been tested. The pH has been measured because it can get influence in the final microgel charge (Table 6.2). pH(o) refers to the initial mixture and pH(f) refers to the last washed.

Entry	$CaCO_3$ [%]	Cross-linker	pH(o)	pH(f)
1	6	Genipin 0.028M	7.25	6.98
2	4	Genipin 0.028M	7.50	7.24
3	4	mTGAse 6%	9.43	7.74

Table 6.2: Summary of experimental conditions.

In the table 6.2 it is observed an increase of pH(o) using mTGase instead of genipin since in pH(f) was not so different so it was decided to use both cross-linkers. Also, two percentages of $CaCO_3$ have been tested. Better results have been obtained with a 4% of microparticles instead of 6% because from 6% nothing has been obtained. It can be explained because the thickness of the template has influence in the physiosorption of the protein and cross-linkers. Gelatin try to get into the pores but how much longer is the path that it has to go throw, more difficult is that it reaches the core and therefore, it is easier that it go out before it has been cross-linked and strengthened.

Before cross-linking two procedures were checked to mix the template with gelatin. The first one consisted of centrifugations and the second one, replacing the centrifugations by sedimentation for 48 hours. However, SEM demonstrates that both procedures lead the same qualitative results (Figure 6.7). The advantage of centrifugation is that is a faster procedure but sedimentation avoids product losses.

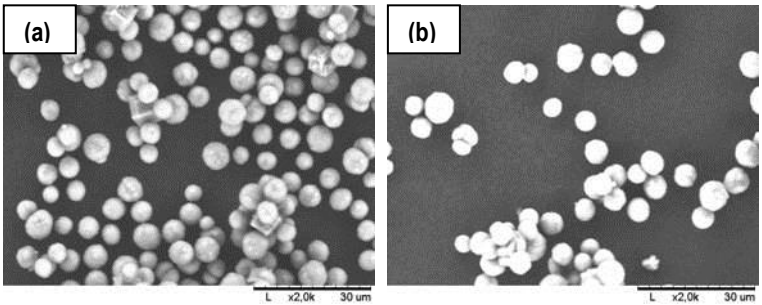

Figure 6.7: SEM micrographs of gelatin into template before cross-linking (a) using centrifugations (b) using sedimentation.

In the Figure 6.8 SEM results can be seen before and after the elimination of the template by EDTA using the method previously reported (Experimental 5.4.1). It seems that good results

have been obtained in all experiments but in image (b) it can be noticed that what it really shows is a broken template, for its bowls and crystals forms, that has not been eliminated as it was expected.

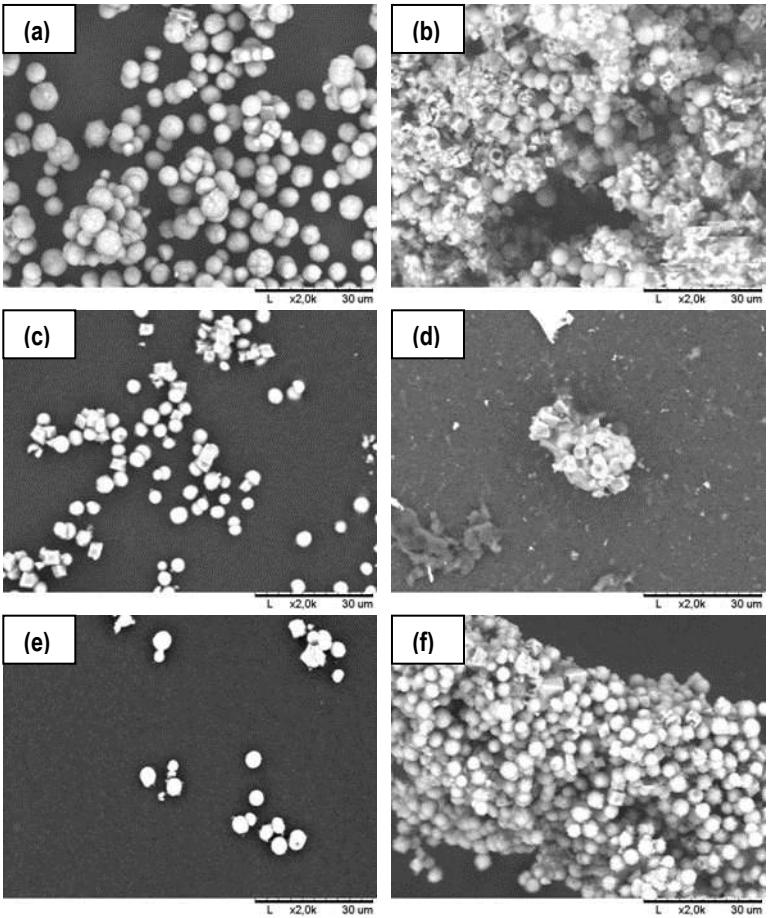

Figure 6.8: SEM micrographs of microgels (a) entry 1 with template (b) entry 1 final microgel (c) entry 2 with template (d) entry 2 final microgel (e) entry 3 with template (f) entry 3 final microgel.

Thermogravimetric analyses (TGA) were carried out to confirm if the template was removed by EDTA treatment at pH 7[13] or it was still present. Figure 6.8 is compared with Figure 6.6 and shows that there is some gelatin in very few proportions (it decomposes between 300-400ºC approximately) but also the presence of the template is confirmed. So, $CaCO_3$ has not been totally removed.

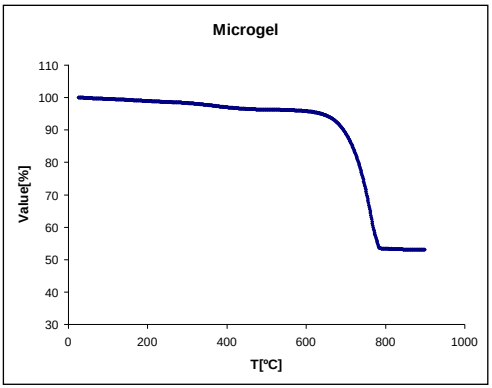

Figure 6.9:TGA of Entry 3 from table 6.2 (4%$CaCO_3$15%Gelatin6%mTGAse)

Therefore, microgel, was stirred for 2 hours with EDTA at pH=11 because being more deprotonated it would be able to complex better with Ca^{2+} and hence, the removal of the template would be more effective. As it can be seen in Figure 6.10, the vast majority of the template has been removed.

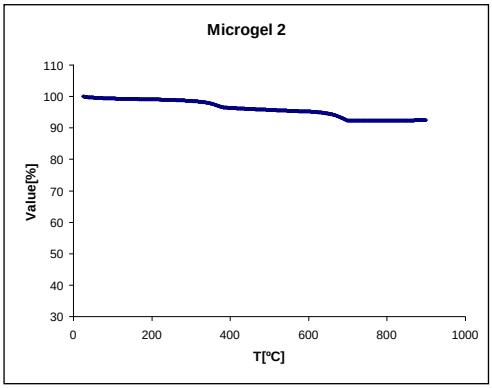

Figure 6.10: TGA of Entry 3 from table 6.2 after stirring 2 hours with EDTA at pH=11.

The biggest disadvantage of this method is that a low amount of microgel is obtained. TGA from figure 6.10 also indicates that the final microgel has only lost 10% of weight so, there were a lot of waste that could not be burned at 800ºC, so unidentifiable.

6.3. PREPARATION OF MICROGELS BY CO-PRECIPITATION

Co-precipitation method allows introducing gelatin into the $CaCO_3$ particles with high amount and using a single step because the template is formed around the protein [14].

In this process, gelatin was labelled with fluorescein (F), dissolved in DMSO at different concentrations (2%, 1% and 0.5%). Good results have been obtained when the concentration of fluorescein was 2% and 1% as can be observed by optical microscopy by fluorescence technique. The Figure 6.11 shows optical microscopy micrographs of a microgel with template using two techniques interferencial contrast (DIC) and fluorescence. As can be seen in Figure 6.11(b,d), gelatin microgel has the desired shape into the template.

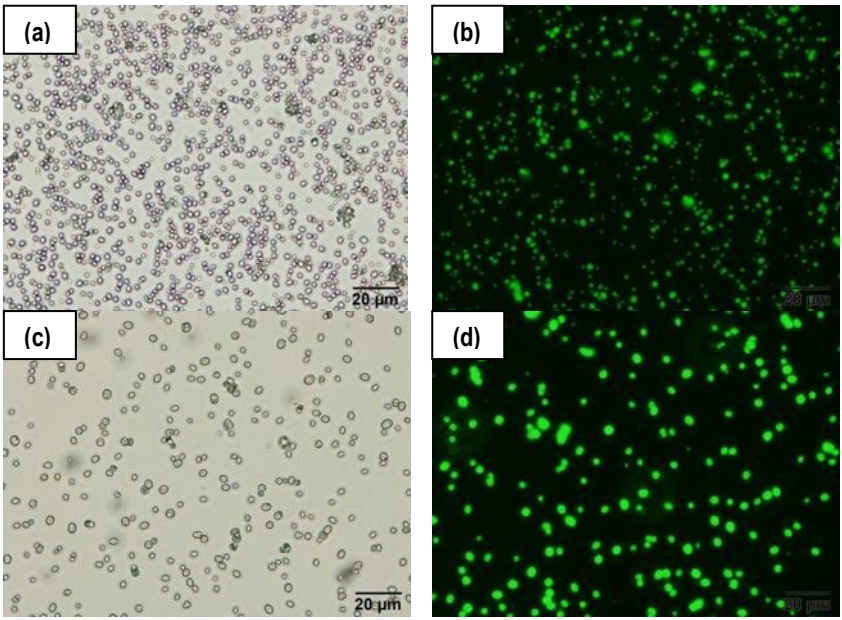

Figure 6.11: Optical microscopy micrographs of microgel with template (a) DIC 1%F (b)fluorescence 1%F (c) DIC 2% F (d) fluorescence 2% F

In Figure 6.12 it can be observed the final microgel once the template has been removed. It is intuited that microgel has been obtained correctly in the desired shape. However, aggregation makes difficult a correct verification.

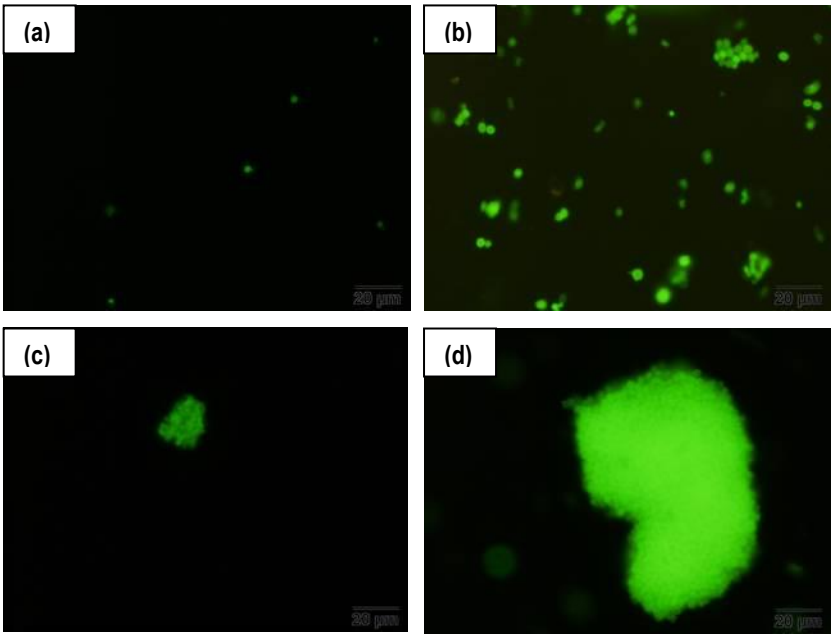

Figure 6.12: Optical microscopy micrographs using fluorescence technique of final microgel without template (a)(c)1% F (b)(d) 2% F

Although, 1% and 2% of fluorescein were used to label gelatin, the concentration of 1% was finally chosen. Both the cross-linker and the fluorescein react with the amino groups of gelatin. The use of 1% of fluorescein would leave more free amino groups which could react with the crosslinker and hence the microgel would be more stable.

In order to obtain a more complete characterization of gelatin microgels, scanning electron micrographs has been carried out (Figure 6.13).

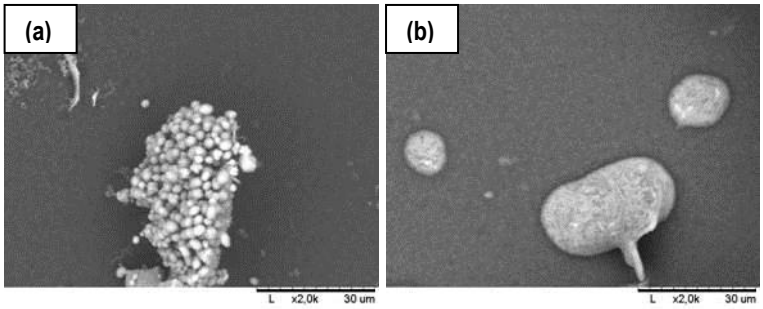

Figure 6.13: SEM micrographs of microgels (a) 1% F (b) 2% F

Two ways were proposed to obtain the solid microgel. The first one proposed consists of filtering by number 3 filter; but the mildness of the microgel causes that it had to be redispersed in a small volume to be able to recuperate it. Then it was frozen with dry-ice and acetone and lyophilized. The second one, instead of filtering the sample, it was placed in an oven for 3 hours at 40ºC to evaporate the solvent.

In Figure 6.14 it can be observed both results. When the solvent is evaporated in an oven (b) aggregation problems are reduced, but flatter image provides less information about structure. Moreover, it looks so similar to the initial $CaCO_3$ microparticles that it does not seem to be a gelatin microgel. Instead, (a) present problems of aggregation but gives information of morphology microgel with hooked gelatin in excess that could not be successfully removed.

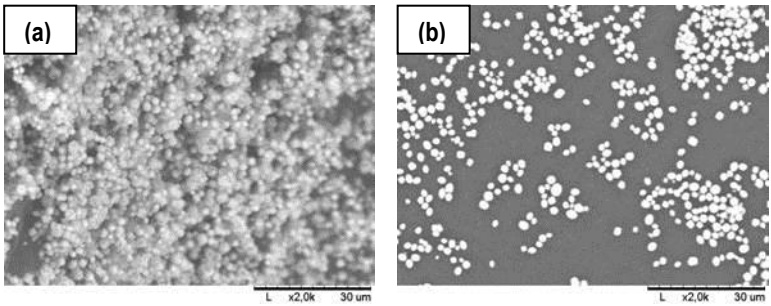

Figure 6.14: SEM micrographs of microgels (a) lyophilized (b) evaporated solvent in an oven

So, as has been already decided, the possibility of filtering had been eliminated because of the mildness of the microgel and the last step was frozen with dry-ice and lyophilized.

To confirm that the template has been eliminated in the final microgel, thermogravimetric analyse (TGA) was carried out. In Figure 6.15 it can be observed the TGA corresponding to gelatin microgel labelled with 1% and 2% of fluorescein and cross-linked with genipin. It can be seen that gelatin discomposes around 200-300 ºC. Moreover, between 350-500ºC there is a weight loss that could correspond to genipin or fluorescein. Besides, between 600-700ºC it can be noticed that there is some template so, then, it has not been completely removed.

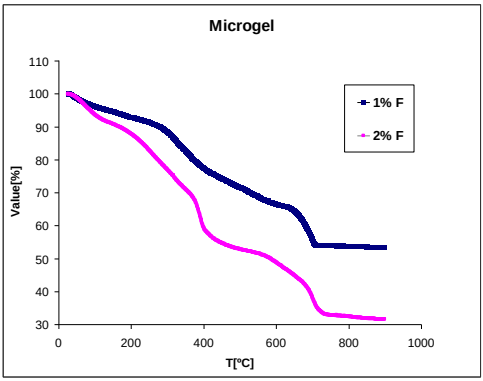

Figure 6.15: TGA of labelled final microgel

When the preparation of the final microgel was finished, their size distribution has been measured from Optical Microscopy micrographs with the program Image J. More than 500 microparticles have been measured. As seen in Figure 6.16, the sample was like $CaCO_3$ if it was compared with Figure 6.3($CaCO_3$ 4 ± 2μm size distribution), with an average size of 4 ± 2 μm.

	Mean	Standard Deviation	Minimum	Median	Maximum
Particle Size [μm]	3.5	1.51	1.25	3.50	5.75
Relative Freqüency	10	9.67	0.24	7.85	26.57

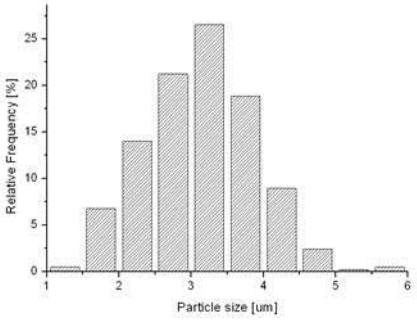

Figure 6.16: Final microgel cross-linked with genipin μm size distribution.

Once the experimental conditions using genipin were optimized, gelatin microgels were also prepared using mTGAse as a cross-linker. Figure 6.17 shows optical microscopy micrographs, using DIC technique. As it can be seen, microgels cross-linked with genipin can be observed. However, when the cross-linker used was mTGAse almost nothing has been observed. This fact makes one suspect that the result obtained in Entry 2 will not be that expected.

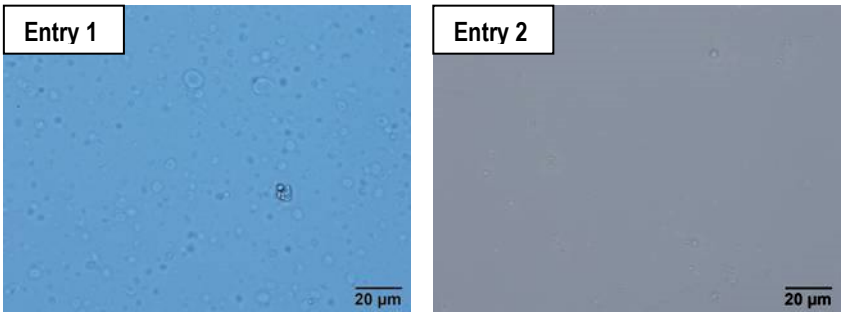

Figure 6.17: Optical Microscopy micrographs by DIC of microgel cross-linked with (Entry 1) genipin (Entry 2) mTGAse

After the last step, both entries have been characterized by SEM and the lyophilized microgel has been successfully obtained in Entry 1 (Figure 6.18), although an excess of gelatin can be observed in some areas. In Entry 2, microgel does not have the expected appearance so, it is suspect that partial charge that mTGAse acquires at pH=11 can influence in microgel formation and stability.

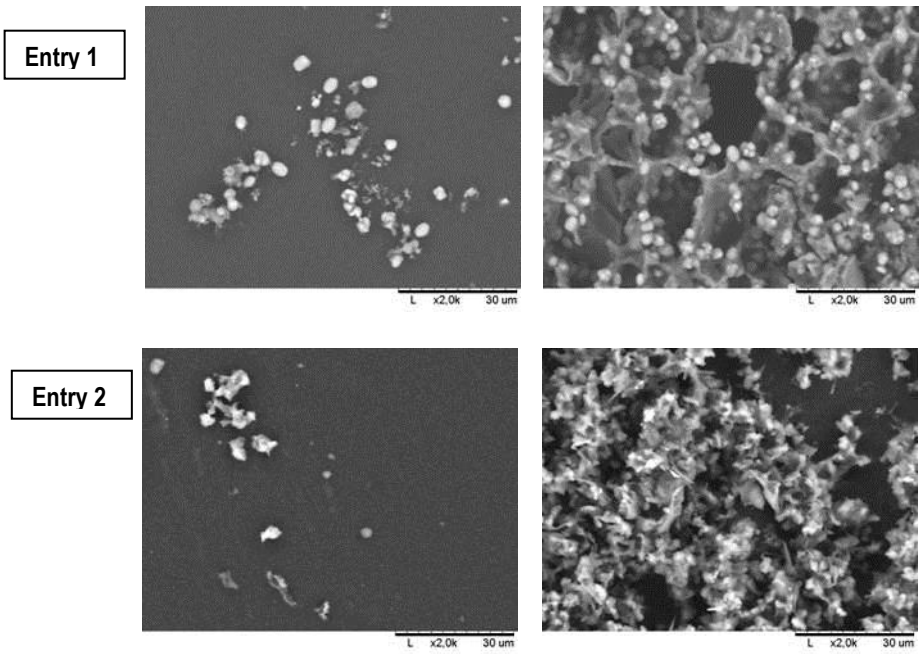

Figure 6.18: SEM micrographs of microgel cross-linked with (Entry 1) genipin (Entry 2) mTGAse

In order to confirm the partial charge of both microgels showed in Figure 6.18, their stability and the hypothesis rainsed on about mTGAse, the zeta potential was measured. The results are showed in Table 6.3.

Entry	Electrophoretic mobility[microcm/Vs]	Cross-linker	ζ[mV]	pH
1	-1.57	Genipin	-20 ± 7	7.40
2	-0.02	mTGAse	-0.2 ± 8	6.86

Table 6.3: zeta potential results.

In Entry 1 from Table 6.3 it is shown that gelatin microgel cross-linked with genipin has a negative surface charge in a neutral pH. It is more or less stable in solution but will trend to coagulate in the future. Entry 2 is a diluted solution of gelatin microgel cross-linked with mTGAse but the result is close to 0 so, very unstable.

Finally, thermogravimetric analyses (TGA) were carried out to confirm that in the final microgel template has been eliminated. Figure 6.19 compares both microgels.

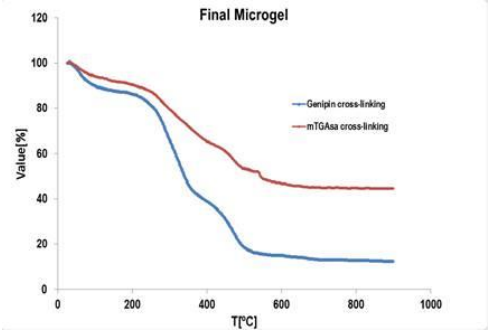

Figure 6.19: TGA of finals microgels.

Blue line corresponds to genipin cross-linked microgel. At first it is appreciated the evaporation of volatile compounds that would correspond to the humidity from the environment. Around 200-300ºC it is observed a large presence of gelatin and about 400ºC it is assumed that the slope change is due to genipin. Also, it is compared with the $CaCO_3$ microparticles blank value showed in Figure 6.6. This demonstrates that there is no presence of the template in the final microgel

Red line corresponds to mTGAse cross-linked microgel. As in the previous case, volatile compounds are appreciated, gelatin too but in less amounts. Moreover, between 400-600ºC there are some slope changes that can be on account of mTGAse and/or instrumental noise due to small amount of sample used, only 0.45 mg. As well as in the previous case, it was demonstrated that there was nothing left from the template but there was something that could not be burned at 800ºC. There was a 45% of weight waste.

Thus, it can be conclude that co-precipitation method is capable of providing about 26 mg of final microgel starting from 2 mL of gelatin 15% and without trace of template. In addition, only the desired morphology has been obtained by cross-linking with genipin due to the possible interference of transglutaminase partial charge. So, probably the microgel cross-linked with mTGAse has not been obtained.

7. CONCLUSIONS

The main objective of this project was to obtain gelatin microgels by a calcium carbonate template and to characterize them. The following conclusions can be drawn from the results obtained:

- The calcium carbonate template has been obtained with the polymorph form of interest, vaterite, in a neutral pH and with a rather low polydispersity. By N_2 sorption technique it has been checked that it was a macropores material with presence of mesopores. Zpotential analysis confirms that it has a low negative surface charge.
- Gelatin microgel prepared by physiosorption presents a lot of problems. It seems that gelatin is not able to reache the core of the template and it easy goes out before the cross-linking. So, quantifiable results could not been obtained, but in SEM micrographs it can be appreciated like bowls or capsules.
- Gelatin microgel prepared by co-precipitation has provided a high amount of product and in a single step. It was a better way because gelatin did not have to cross the template and got into, the template is formed around it. Gelatin microgel has a size distribution similar to $CaCO_3$ microparticles. Zeta potential analysis rebels that when genipin is used as a cross-linker, the microgel had a slight negative charge in solution. When mTGAsa is used as a cross-linker, a value very close to 0 was obtained and so, bad results has been obtained from it.

8. FUTURE WORK

In a future work it would be interesting:

- To try both methods, physiosorption and co-precipitation, using different concentrations of gelatin (in this work it is used a 3.5mM concentration).
- To prepare microgels with other polymers and/or carbohydrates such as chitosan, using calcium carbonate as a template.
- To make a more detailed study using mTGAse as a cross-linker to observe the factors that influence in microgels cross-linking.
- To use a more ecofriendly method to eliminate the template, as could be Ascorbic Acid.

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10. ACRONYMS

BET: Brunauer–Emmett–Teller theory

BJH: Barrett-Joyner-Halenda theory

DIC: Differential interference Contrast

DMSO: dymethil sulfoxide

EDTA: Ethylenediaminetetraacetic acid

F: fluorescein

G: gelatin

mTGAse: microbial transglutaminase

PBS: Phosphate buffered saline

PSS: Anionic sulfonated polystyrene

SEM : Scanning Electron Microscopy

TGA: Thermogravimetric Analysis

tTGAse: mammalin tissue transglutaminase