Exploring Synthesis Methods of Alginate/Gelatine Microgels

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Abbreviation

AlMA: alginate methacrylate.

GelMA: gelatine methacryloyl.

rpm: revolutions per minute.

xG: times gravity.

%wt: weight percent.

w/v: weight per volume.

Foreword

Absolutely extraordinary. That is how I would describe my first working experience abroad.

I would like to express my gratitude to Joe Forth for his gracious welcome, his confidence in me, his assistance, and the autonomy he granted during this work placement. This experience has not only enriched my academic journey but also shaped my personal growth. The interactions with the Liverpool community have been particularly enriching, especially those with my colleagues. I am grateful to Joe Bathe for his assistance in introducing me to the synthesis of hydrogels. His technical and chemical expertise as the ones of Sara Silva and Cameron Beaney, were also instrumental in the success of the work. I would like to acknowledge Alex Ciupa for the unrestricted access to the formulation laboratory at the Material Innovation Factory© he provided me, with his technical assistance. Pedro Oseliero Filho's expertise in microgel sizing was also of a great help. Finally, thanks to Athina Nikalaou's support throughout my rheological studies.

It all began more than a year ago with an unsolicited application to Sir Munir Pirmohamed[1] and an interview with David Dickens[2] and Joe Forth. And then, at the end of a tunnel of long administrative hurdles, the first day of the rest of my life.

[1] Director of the Centre for Drug Safety Sciences at the University of Liverpool.
[2] Lecturer in the Department of Molecular and Clinical Pharmacology at the University of Liverpool.

I. Introduction

My internship took place within Dr Forth's group, which is part of the Department of Chemistry in the School of Physical Sciences at the University of Liverpool. This new group focuses on the synthesis of micro-hydrogels (microgels) for therapeutic purposes. These objective concerns tissue engineering applications [1] as ink and/or a support for 3D printing vascular structures, forming a scaffold where cells can be cultured.

My study aimed to produce and investigate the properties of biocompatible microgels composed of gelatine and sodium alginate [2].

A hydrogel is a material primarily made of water, with a three-dimensional polymeric structure capable of retaining a large amount of water within its network. The polymer chains are typically hydrophilic, these chains form a three-dimensional network that creates spaces, or pores, where water can be trapped. The water is absorbed through hydrogen bonding between the water molecules and the hydrophilic groups in the polymer. This ability to absorb and retain water allows hydrogels to swell and maintain a hydrated state, making them ideal for applications such as wound dressings, drug delivery systems, and tissue engineering. [3] (Figure 1). Hydrogels are characterized by their ability to swell and change shape or volume in response to external stimuli, such as temperature, pH, or ion concentration [4]. Microgels are thus micrometric spherical particles of hydrogels, the interest of which is explained by their injectability[3] and increased porosity[4](Figure 2) [5].

[3] Being fluid, it provides an increased contact surface (*e.g.*, in the case of a wound) and the ability to reach difficult-to-access areas.

[4] Better diffusion of chemical elements and cell culture.

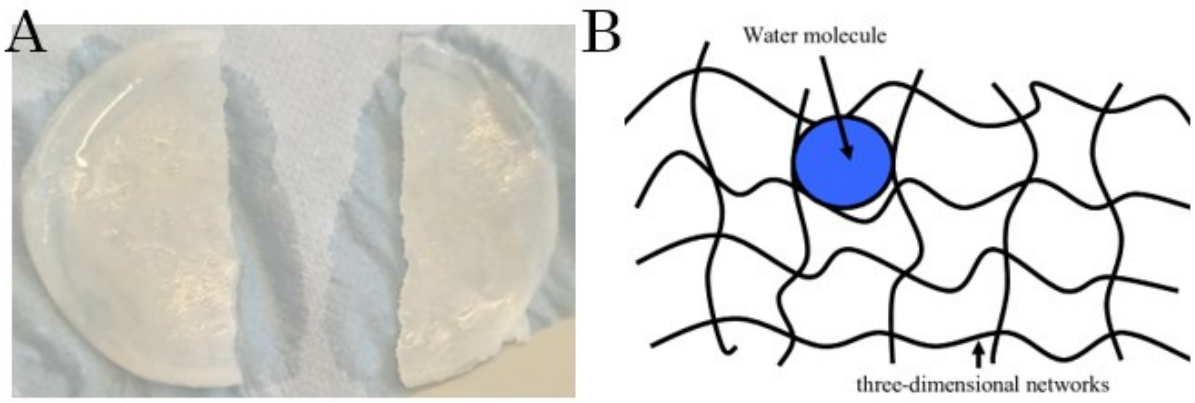

Figure 1: (A) Picture of an alginate and gelatine-based hydrogel obtained. (B) Hydrogel structure representation from Darwis and Darmawan, Atom Indonesia, 2010.

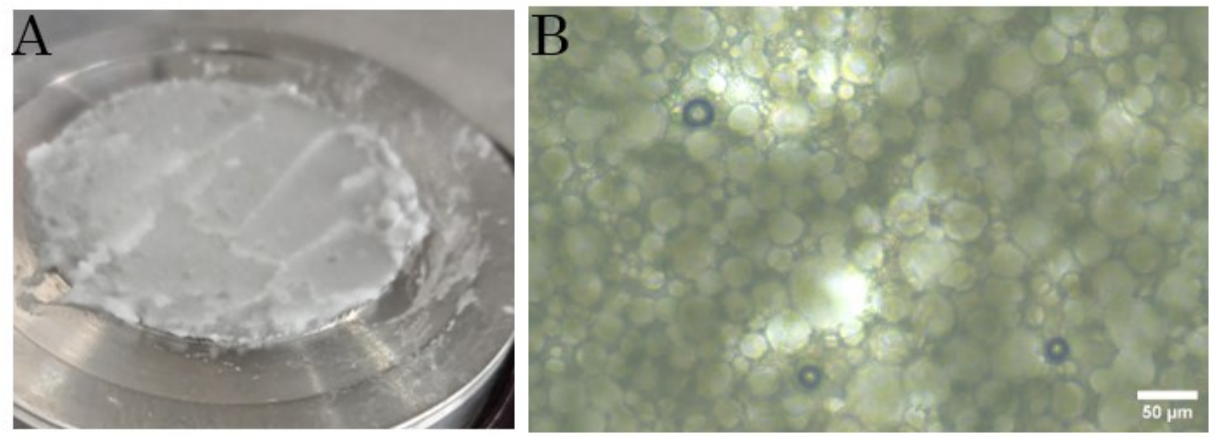

Figure 2: Macroscopic (A) and microscopic (B) pictures of microgels made of gelatine (8% wt). The more fluid aspect (possible spreading) of the microgels contrasts with the rigidity of the hydrogels shown in the previous figure.

The study conducted by Chang *et al.* [6] highlights the interest in hydrogels composed of gelatine and alginate. It has been demonstrated that these hydrogels exhibit a relatively low degradation rate *in vitro*, allowing them to maintain their structure for several weeks while supporting cell proliferation.

Gelatine is an animal-derived protein obtained by partial hydrolysis of collagen, primarily extracted from the bones and skin of cattle, pigs, or fish. Its chemical structure consists of polypeptide chains which contains amino acids such as glycine (majority), proline and hydroxyproline [7] (Figure 3). Hydrophilic groups such as hydroxyl (-OH) and amino (-NH_2) groups allows it to form gels in an aqueous solution when heated and then cooled. At room temperature, gelatine forms an elastic and translucent gel.

Alginate is a natural polysaccharide extracted from brown seaweeds, composed of units of D-mannuronic acid (M) and L-guluronic acid (G) with hydrophilic carboxyl groups (Figure 4). In contrast to gelatine, alginate is not degradable by mammals [2][8], making this component therapeutically interesting.

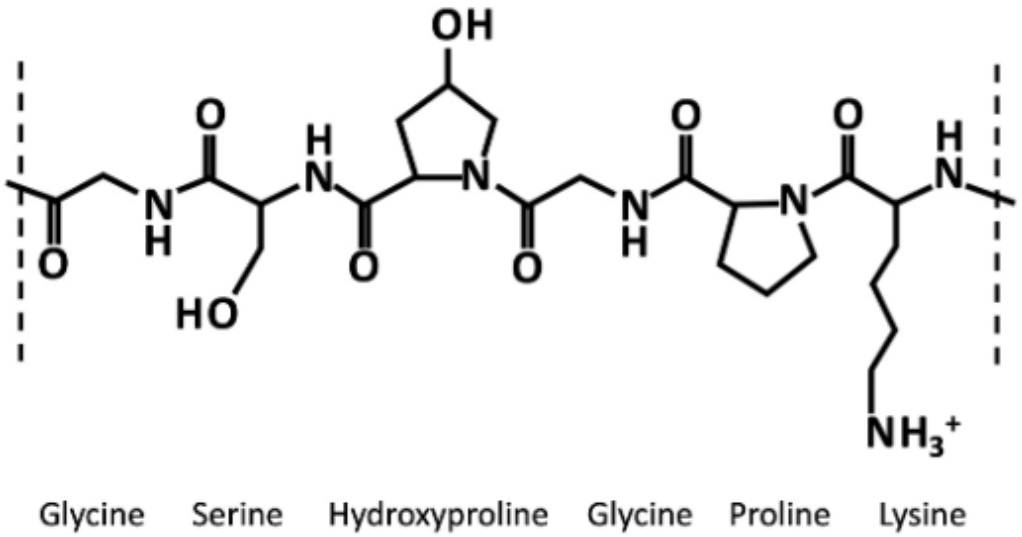

Figure 3: Gelatine chemical structure, highlighting amino acids. From Milano *et al.*, Pharmaceutics, 2023.

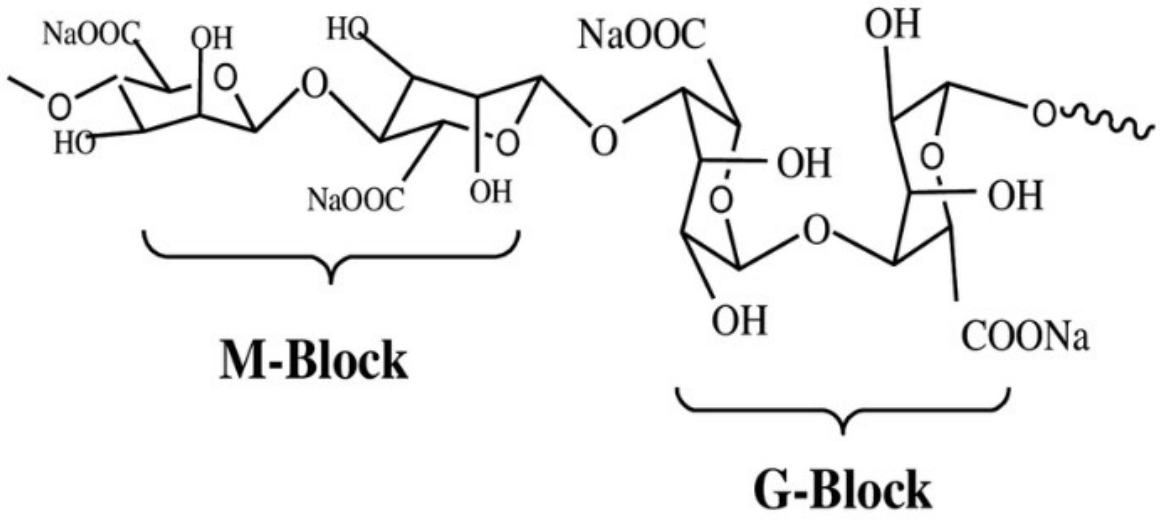

Figure 4: Alginate chemical structure with M and G blocks. Salisu *et al.*, Desalinisation and water treatment, 2016.

This study focuses on microgels rather than bulk hydrogels due to their unique properties, as briefly introduced earlier. Their yield-stress and shear-thinning rheology, explained by

the freedom of movement of each particle, facilitates their use as a precise bioink [9]. This ink can either be used directly or serve as a support for sacrificial ink. The overarching goal is to produce microgels that can act as suspensions that support the 3D printing of delicate structures, such as fine vascular structures (micrometric scale) where cells can be cultured. Beyond this immediate application, microgels can be used directly to heal wounds due to their porosity, by deliveries of encapsuled drugs [10] or tissue regeneration, where the cell migration efficiency is improved [11].Thus, the goals were: (i) establishing a protocol for obtaining microgels composed of gelatine and alginate, (ii) characterizing them in terms of diameter with an arbitrary target set at 5 μm^5 and (iii) studying their rheology.

In order to achieve the objectives outlined above, the study initially focused on the protocol established in the group, that led to the production of microgels composed of gelatine methacryloyl (GelMA) . The first task was to verify whether this protocol is valid in the presence of gelatine and alginate. The answer to the previous question is negative, leading to the development of a new protocol based on the existing knowledge within the laboratory and on the literature. The second task was to characterize the products obtained throughout the synthesis process *i.e.* during the emulsion and after the washings. This was achieved through the analysis of the appearance of the products as well as rheological studies.

Following an overview of the host laboratory's structure, the equipment used and the methods employed will be outlined. This report will conclude with a critical analysis of the results obtained.

II. The Forth Group at the University of Liverpool

The University of Liverpool, founded in 1881, is one of the leading institutions in the UK as member of the prestigious Russell Group of research-intensive universities. The Department of Chemistry (Figure 5), where my internship took place, is renowned for its commitment to cutting-edge research[6]. Since late 2017, the public university has been home to the Materials Innovation Factory (MIF), a world-class research facility dedicated to advancing the development of new materials. Co-founded by the University of Liverpool, Unilever ™, the Sir Henry Royce Institute and the UK's National Institute for advanced materials research and innovation; the MIF brings together scientists, engineers, and industry experts to drive innovation in fields such as energy, electronics, and healthcare.

Dr Joe Forth is a lecturer with a joint appointment at the Departments of Physics and Chemistry at the University of Liverpool with a background in Soft Matter Physics, Nanomedicine, Neural Drug Delivery, and 3D-Printing. He undertook his PhD in Physics in the group of Paul Clegg and Wilson Poon at the University of Edinburgh and held postdoctoral fellowships at Lawrence Berkeley National Laboratory (Prof. Tom Russell, Dr. Brett Helms), and UCL (Prof. Giuseppe Battaglia). He later held the Ramsay Memorial Fellowship [7]at UCL.

Founded just over a year ago, the Forth Group focuses on soft matter with the motto: *Πάντα ῥεῖ* [8]. During my internship, the laboratory had four other permanent members, including three PhD candidates. Joe Bathe (gelatine-alginate based hydrogels), Cameron Beaney (GelMA microgels for 3D printing) and Emmanuel Ombo (automated materials

[6] 3rd in the UK for outstanding research impact (Research Excellence Framework 2021).
[7] Offered to postdoctoral chemists who already have some postdoctoral experience of research but who are in the early stages of their career to initiate a programme of original and independent research.
[8] Same motto as The Society of Rheology © . Translate as 'everything flows', from Heraclitus (6th – 5th century BC). According to him, the universe is in constant change and that nothing remains the same. It emphasized the concept of perpetual motion and transformation.

chemistry). Dr Sara Silva, visiting researcher from the University of Porto (Portugal), focused on 3D printing (vascular patterns on sacrificial bath of Carbopol ®).

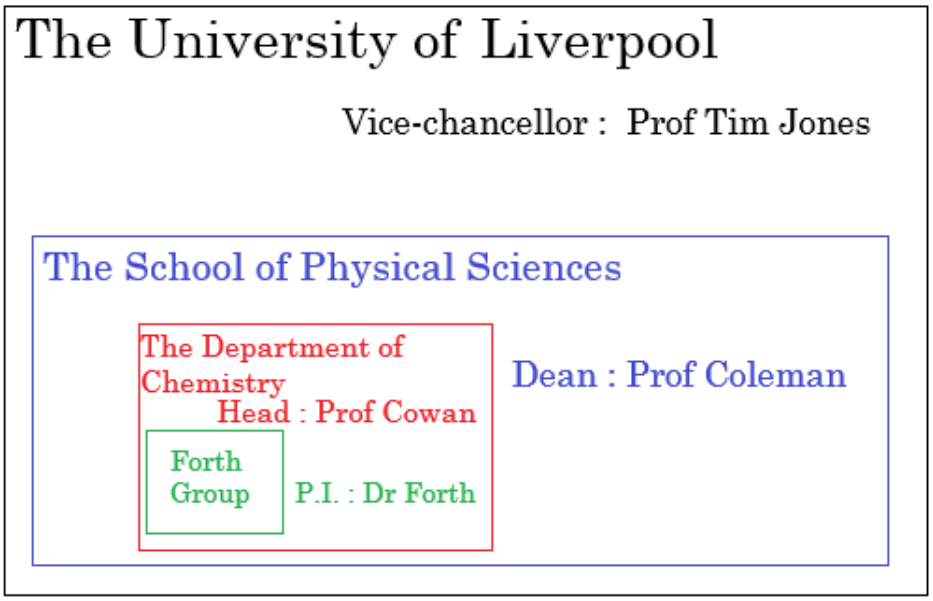

Figure 5: Administrative situation of the Forth Group at the University of Liverpool.

III. Materials and methods

The goals were: (i) developing a protocol to produce gelatine-alginate microgels, (ii) analysing the products at each step of the synthesis and (iii) make rheological studies.

III.1. Hydrogel synthesis

Gelatine (type A, G1890) and sodium alginate (W201502) were used in their raw form (Merck™). GelMA and alginate methacrylate (AlMA) were obtained after synthesis from gelatine and alginate, respectively, by the addition of methacrylate anhydride (Merck™).

III.1.1. Synthesis of gelatine methacryloyl

Gelatine methacryloyl (GelMA) was synthesized using a simplified one-pot method [12]. A sodium carbonate bicarbonate buffer was prepared by dissolving 0.318 g of sodium carbonate and 0.586 g of Sodium Bicarbonate in 100 mL of Milli-Q® water to obtain a 0.1 M solution. This buffer was heated to 50°C under continuous stirring at 500 rpm. Gelatine was then added to achieve a final concentration of 9.1% (w/v), and the pH was adjusted to 9.0 using 5M HCl and 5M NaOH solutions. This pH above gelatine's isoelectric point, ensures that the free amino groups of lysine remain neutral, facilitating their reaction with methacrylic anhydride [12] (Figure 6B).

After ensuring the gelatine was completely dissolved, methacrylic anhydride (0.2 mL per gram of gelatine) was slowly added to the solution. The reaction mixture was maintained at 50°C and stirred at 500 rpm for 2 hours. The pH was checked every 30 minutes to monitor the progress. After 2 hours, it was adjusted to 7.4 to stop the reaction to maintains its molecular integrity, preventing denaturation or changes in its functional properties.

III.1.2. Synthesis of alginate methacrylate

The synthesis of AlMA following Hassany *et al.* [13] (Figure 6A), was carried out at 4°C, with the addition of methacrylic anhydride (5-fold molar excess) proceeding slowly over 24 hours at a rate of 0.5 mL per hour, with a pause halfway through the process. The pH was here put at 8, above the sodium alginate isoelectric point [14] to maintain the

negative charge on the -COO groups. This contributes to the radical-initiated addition reactions that occur to form AlMA, involving the alginate hydroxyl groups and the methacryloyl groups from methacrylic anhydride [13]. The low temperature and slow addition rate of methacrylic anhydride help to counteract the instability of the radicals, which is an intrinsic characteristic of them. GelMA and AlMA were characterized using Proton NMR (Brucker™) in comparison with the literature.

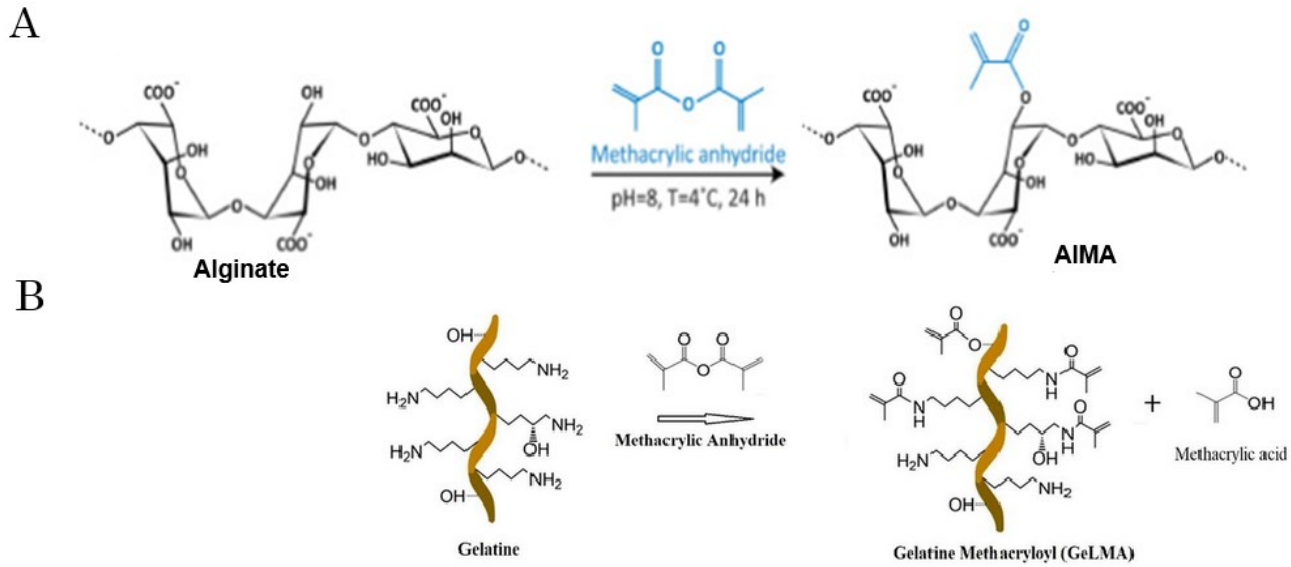

Figure 6 : (A) AlMA synthesis. From [13] (B) GelMA synthesis. From Dorterler *et al.*, Polymers, 2024.

The resulting liquids were transferred into dialysis bags and dialyzed against Milli-Q® water at 50°C and stirred at 350 rpm. The water was replaced at intervals of 24 hours over a total duration of 4 days. Dialysis at 50°C was necessary to avoid gel formation or splitting of the dialysis bags. Once dialysis was complete, the product was filtered through a sterile 0.22 µm filter (Millipore® Steriflip® from Merck™), frozen in a freezer at -20°C, and lyophilized over 2 days. The final dried GelMA was stored at -20°C.

Three different aqueous solvents for the hydrogels' synthesis were studied: sodium hydrogen carbonate (100 mM), PBS, and Milli-Q® water. The first two were prepared by dissolution, one tablet of Sigma Aldrich™ PBS in 200 mL of Milli-Q® water, sodium hydrogen carbonate granules from Merck™).

III.1.3. Hydrogels synthesis method

Hydrogels were synthesized following the protocol of Chang *et al.* [6]. In a 25mL conical flask, after adding the solvent (Milli-Q® water or 0.1M sodium hydrogen carbonate solution or PBS) the solutes are added one by one after dissolution (heating at 50°C, stirring at 800 to 1300 rpm depending on the solvent used[9]). In the context of using photosensitive chemical elements *i.e.* GelMA and AlMA, a photoinitiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), was added (2.5% of the mass of GelMA and AlMA [10]) to induce physical crosslinking. The hydrogels are stored at room temperature and are thereafter identified as bulk, as opposed to their modification into microgels.

III.2. Microgel synthesis method

The microgels were formed by first making water-in-oil emulsions[11] in a batch process. The aqueous phase (dispersed) corresponds to the hydrogel. The immiscibility between the two phases (aqueous polar phase and apolar oily phase) leads to the formation of hydrogel spheres within the oil phase (mineral oil and sorbitan monooleate at 2% wt, both from Sigma Aldrich™). In certain cases, described in the following sections, crosslinking of the polymers has occurred to enhance the stability of the formed droplets.

This conformation as spherical droplets is explained by the surface tension between the two immiscible phases [15]. The molecules of the aqueous phase located at the interface with the oil experience a force directed inward toward the aqueous phase (hydrogen bonding); resulting from the asymmetry between the aqueous phase (a donor and acceptor of hydrogen bonds and polar) and the oil phase, which is apolar and not involved in hydrogen bonding. This three-dimensional "inward pull" thus leads to the formation of spherical droplets [16].

The stabilization of these droplets was ensured by sorbitan monooleate (Span® 80), a surfactant whose hydrophilic heads and hydrophobic tails form micelles (Figures 7 and 8). More precisely, the surfactant is adsorbed at the interface. The hydrophilic heads form hydrogen bonds, stabilizing the interfacial aqueous molecules, while the hydrophobic tails align through Van der Waals interactions within the oil phase. This barrier prevents the coalescence of droplets that could otherwise form particles exceeding the micrometric

[9] The dissolving power of the sodium hydrogen carbonate solution is lower.
[10] According to the laboratory protocol.
[11] By definition, a mixture between two immiscible phases.
.

scale. The specific use of this surfactant is explained by its low Hydrophilic-Lipophilic Balance (HLB), which makes it lipophilic and therefore highly soluble in oil, making it particularly suitable for water-in-oil emulsions, where oil serves as the matrix [17].

The established protocol follows that of Muir *et al.* [18], 3 mL of hydrogel (preheated to 50°C to become liquid) are added drop by drop to 27 mL of the oil phase in a conical flask, while continuously stirring and heating (800 rpm, 50°C). The droplets are formed using a needle (Fisnar QuantX 8001079) connected to a 3 mL syringe (luer lock), where the operator applies a light pressure.

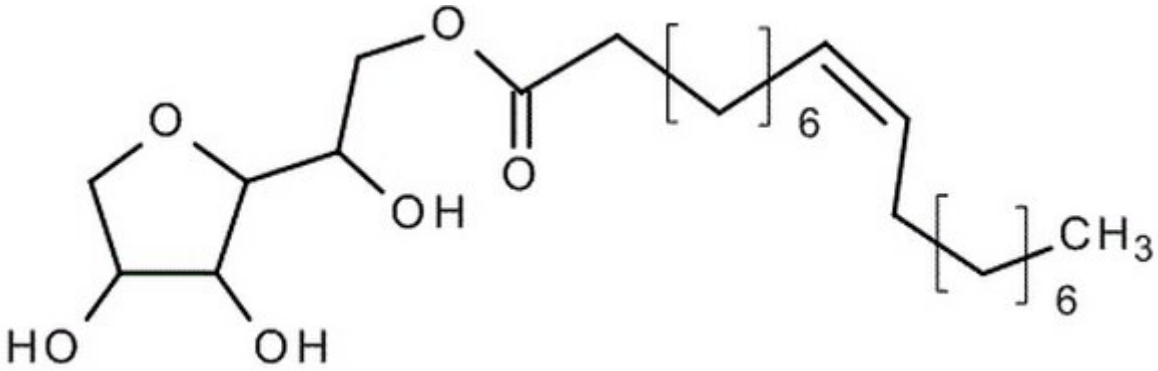

Figure 7: Span® 80 chemical structure. From Merck™'s official website.

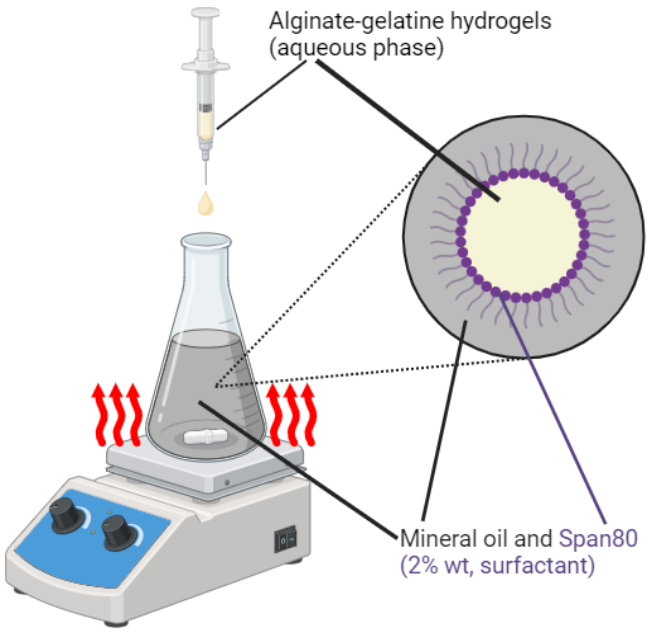

Figure 8: Schematic representation of a microgel synthesis. Made with BioRender©.

In the presence of GelMA and/or AlMA, an ultraviolet radiation (405 nm) phase follows the emulsion. This exposure to UV light[12] triggers the crosslinking of the polymers. Crosslinking is a process in which polymer chains are chemically bonded together, forming a network structure. When exposed to UV light, the photoinitiator activates, initiating a reaction that creates covalent bonds between the GelMA and AlMA polymer chains (Figure 9). This process significantly enhances the stability of the microgels [18].

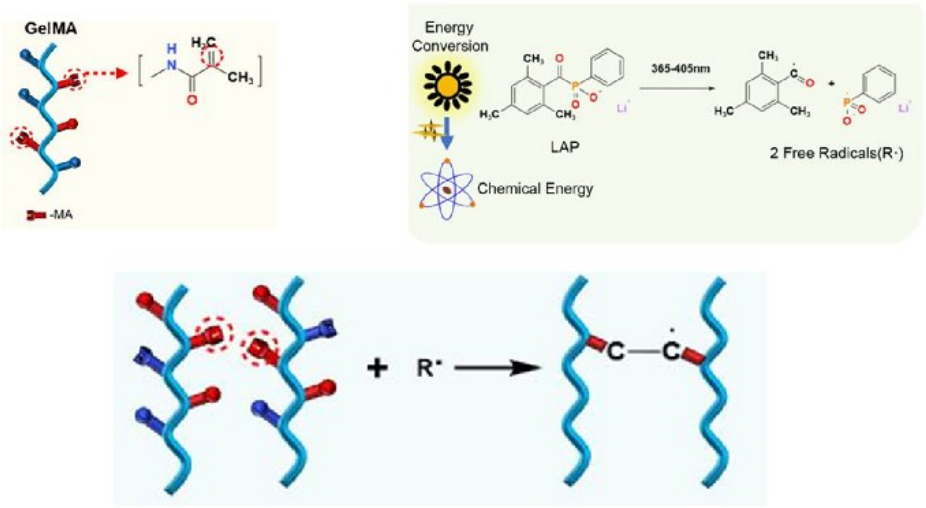

Figure 9: Simplified mechanism of GelMA crosslinking (applicable to AlMA). The modified polymers are linked by free radicals. From Jing He *et al.*, Advanced Healthcare Materials, 2023.

Transferred into a 15 mL Falcon™ tube, the emulsion is first centrifuged at 4500 xG (4°C[13]) in accordance with [19], to separate the particles from the oil phase. Once the oil is removed, the product is washed ten times (centrifugation at 2000 rpm, 4°C) using two different methods.

On one hand, with acetone, which requires a minimum drying time of 24 hours and subsequent rehydration. This rehydration was performed either with PBS or with a filler phase[14]. Acetone is a polar organic solvent that is also partially miscible with nonpolar substances, making it highly effective at dissolving mineral oil. Its amphiphilic nature

[12] 10 minutes are sufficient in presence of 4% wt of GelMA.
[13] Parameters empirically maintained to stabilize the emulsion.
[14] Aqueous phase of PBS containing the components of the hydrogels in very low amounts (1%wt).

allows it to disrupt the cohesive interactions within the oil, breaking it down into smaller components that can be removed from the sample surface [20].

On the other hand, a washing step with PBS was performed [18], with or without a prior wash with PBS and Tween 20® at 1% wt (Sigma Aldrich™). After the final wash, the contents of the tube were filtered (Millipore® Stericup® from Merck™). Phosphate-buffered saline (PBS) can contribute to the removal of oil residues despite not being a strong solvent for hydrophobic substances. Its ionic composition helps disrupt weak interactions between oil and sample surfaces, particularly if the surface has charged or polar regions. PBS also aids in mechanically dislodging oil droplets or thin layers through rinsing or agitation. Tween 20 ® add to PBS enhances the removal of residual oil. The surfactant reduces the interfacial tension between the hydrophobic oil and the aqueous PBS solution, allowing for the emulsification of oil droplets.

III.3. Product characterization

III.3.1. Attempts to develop an image analysis algorithm to extract droplets and microgels' size distributions

A Python® code was implemented to study the diameter of the particles using the Hough transform [21]. However, it was not possible to obtain robust data due to the non-detection of the smallest particles (Appendix A1). This was explained by the following numerous parameters of the code that needed to be adjusted empirically.

The Hough transform is used in image processing to detect geometric shapes, such as circles, by transforming image points into a parameter space. For circle detection, it identifies patterns in an image corresponding to the equation $(x-a)^2+(y-b)^2=\boldsymbol{r^2}$ where (a,b) represents the circle center and r its radius. Each edge point in the image votes for potential circles that could pass through it, and these votes are recorded in an accumulator array. Using OpenCV's *cv2.HoughCircles* function, the method detects circles based on parameters[15] like the resolution ratio, minimum distance between circle centers, and thresholds for edge detection and circle confirmation. Additional settings, such as the minimum and maximum radius, refine the detection. This process is robust and effective even in noisy images, but it can be computationally intensive and requires careful parameter tuning to ensure accurate results. Indeed, as mentioned in the results section, the program has difficulty detecting the smallest particles. A future perspective is

[15] Parameters empirically adjusted.

therefore to determine the appropriate parameters to ensure results that account for all particles, regardless of their size.

All the size studies were thus conducted manually.

III.3.2 Rheological properties

The ARES-G2 rheometer (TA Instruments®) was used at the Material Innovation Factory©. Rheological tests are essential for understanding the viscoelastic properties of materials. Two common tests were performed.

The flow sweep measures how the viscosity of a material changes with varying shear rates. It provides valuable information about the flow behaviour, helping to determine whether a material behaves like a liquid or a solid under different stress conditions. The amplitude sweep test, on the other hand, examines the material's response to different strain amplitudes. It helps to identify the material's linear viscoelastic region, where it behaves consistently under applied strain, and the non-linear region, where the material starts to deform permanently. Together, these tests provide comprehensive insights into the material's behaviour to evaluate its use as direct bioink and/or sacrificial bath.

IV. Results and discussion

IV.1. Emulsions made of micrometric hydrogel particles

The first step involved the study of the initial stage of synthesis, *i.e.* during the emulsion. The microscopic images (Figure 10) show the formation of micrometric particles with approximately diameters of 50 µm or less.

It seems that the increase of gelatine leads to smaller particles. The amphiphilic nature of gelatine could provide an explanation. Indeed, as it is composed of hydrophilic (lysine, serine) and hydrophobic (proline, hydroxyproline) amino acids [22], its behaviour resembles that of a co-surfactant, thus allowing for greater stability[16]. Reducing the amount of gelatine and increasing the proportion of alginate (non-amphiphilic) [23] would therefore increase the interfacial tension, leading to a tendency for droplet coalescence [24]. To gain in stability the system must minimize the droplets surface tension, so their surfaces between the immiscible phases. To achieve that, the coalescence of droplets leads to one bigger droplet with a smaller interface surface than the sum of the droplets' ones.

A polydispersity in particle sizes has been observed, recognized as an intrinsic limitation of the batch emulsion technique used [18]. To obtain an emulsion consisting of smaller droplets (with a diameter of approximately 5 µm), the agitation speed was adjusted but without success. It would therefore seem advisable to test the use of finer needles, allowing for the addition of smaller quantities of hydrogel during the emulsification process.

[16] Explained in III.2.

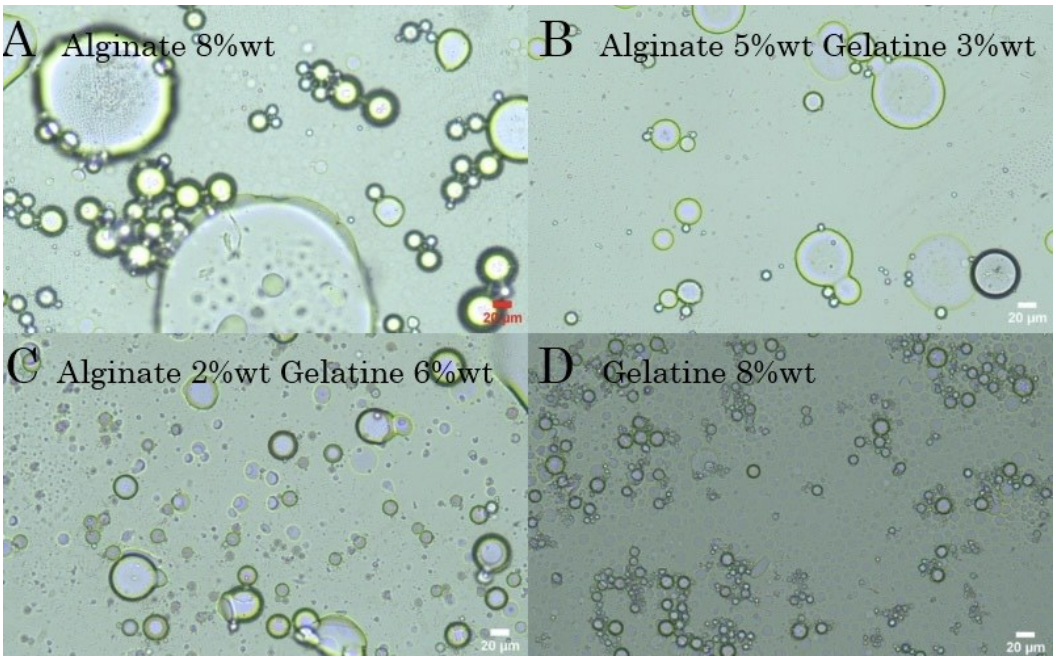

Figure 10: Microscopic images of hydrogels in oil emulsion in batch process composed of (A) Alginate 8%wt (B) Alginate 5%wt and Gelatine 3%wt (C) Alginate 2%wt and Gelatine 6%wt (D) Gelatine 8%wt.

IV.2. Gelatine microgel characterization

After the emulsion, washing the particles constituted the second and final step in obtaining microgels. Similar to the previous section, the objective was therefore to characterize the particles obtained at this stage.

Hydrogels composed exclusively of gelatine were used as a "positive control" to establish the protocol presented in the previous section. This choice was explained by the ease of their preparation (higher solubility compared to alginate) and previous work within the laboratory that led to the formation of microgels made from GelMA. Preliminary results indicate that microgels composed of an aqueous phase of $NaHCO_3$ were unable to form a pellet during washing. This was not the case when Milli-Q® water replaced the sodium bicarbonate solution (Figure 11). The washes were thus successfully carried out, leading to the synthesis of microgels made from gelatine (Figures 12 and 13).

As the washings and rehydration were carried out respectively at 4°C and at room temperature (solid gelatine), the absence of a pellet during washing in PBS and acetone can be attributed to the ionic strength provided by the $NaHCO_3$ solvent. Indeed, as shown by Andersen *et al.* [25], ionic strength causes a decrease in the melting point of hydrogels

composed of gelatine. It is therefore possible that the pellet liquefied at a temperature where it would remain solid in the presence of non-ionic Milli-Q® water.

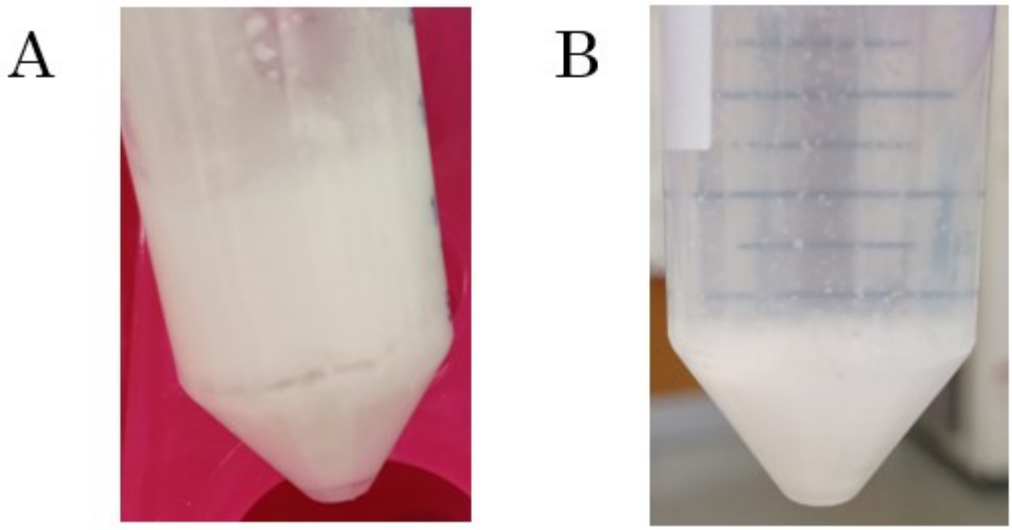

Figure 11: Illustration of the difference observed in the laboratory between the non-sedimentation of microgels composed of $NaHCO_3$ (A) and the sedimentation of microgels composed of Milli-Q® water (B), facilitating their washings in PBS.

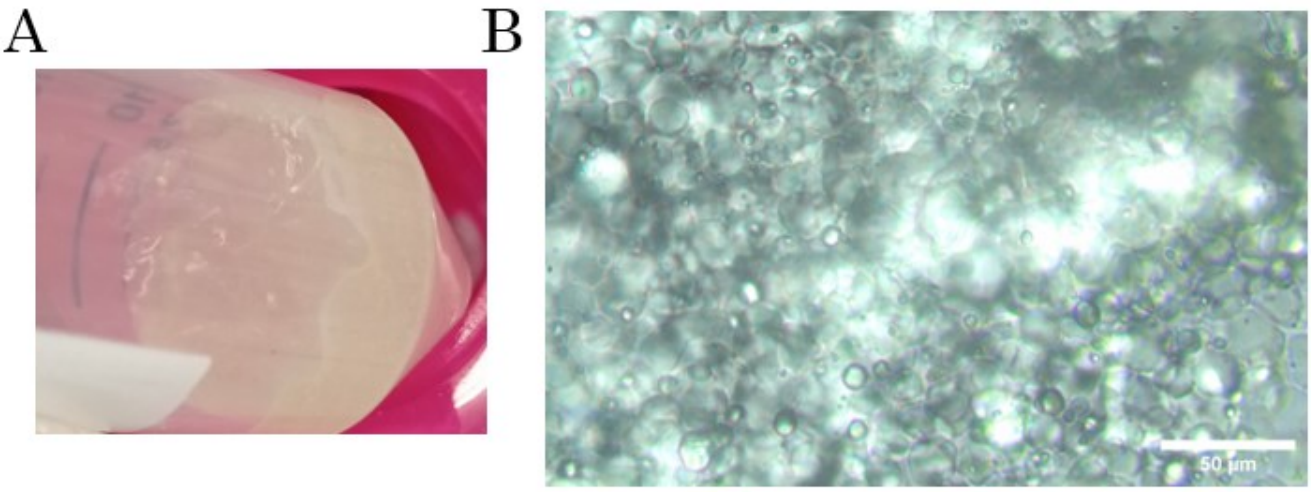

Figure 12: Macroscopic image (A) and microscopic image (B) of microgels composed of 8% wt gelatine after washings in acetone, evaporation, and 24-hour rehydration in PBS.

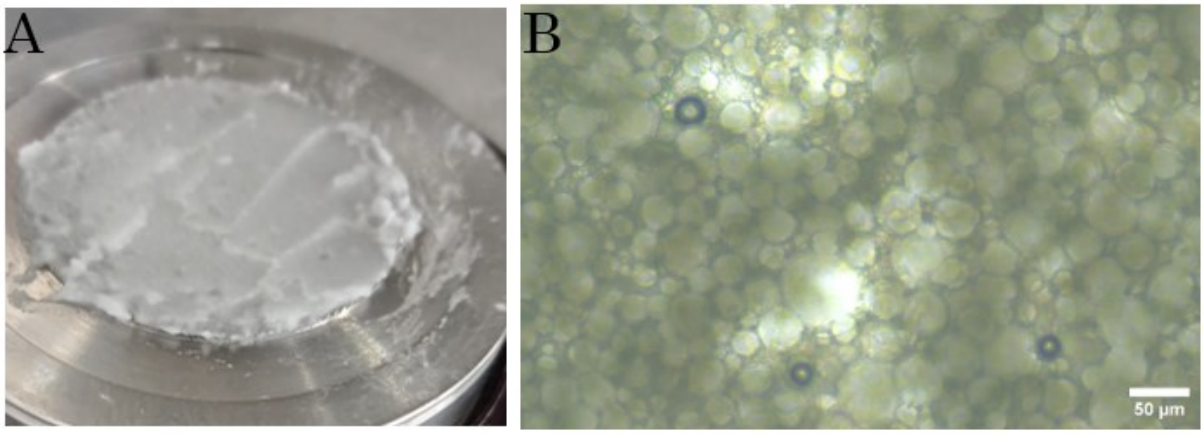

Figure 13: Macroscopic (A) and microscopic (B) pictures of microgels made of gelatine (8% wt) washed in PBS.

The microgels washed with acetone exhibit a diameter of 30 µm in almost all cases (asymmetric distribution), whereas their diameter is approximately 19 µm after PBS washing, with a symmetric distribution (Figure 14). Unlike washing with PBS, hydrogels washed with acetone (and thus dried) swell due to hydrophilicity when immersed in PBS for 24 hours.

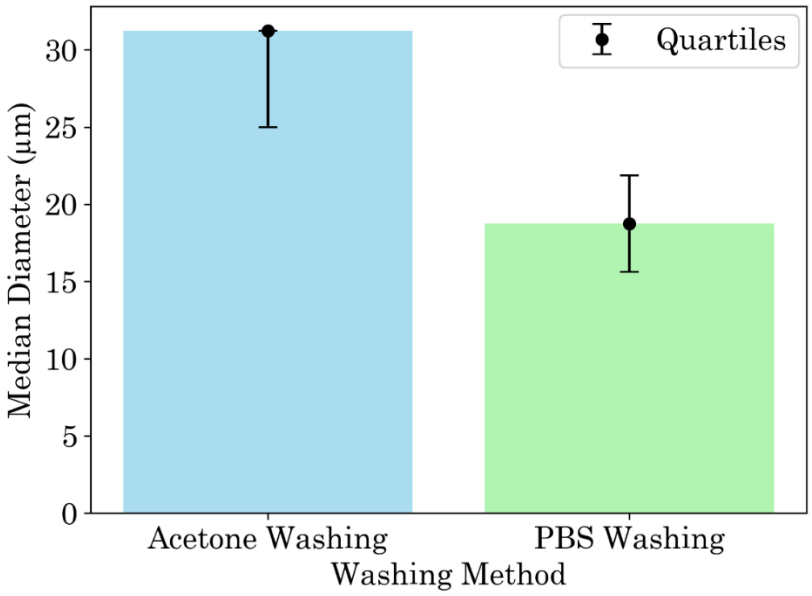

Figure 14: Distribution of microgels diameters composed of gelatine (8% wt) based on the washing method used (after rehydration for the acetone one). Measurements were made manually.

IV.3. Alginate leads to the coalescence of microgels

Previously, microgels composed exclusively of gelatine were obtained and characterized. The next objective was to add alginate, thereby reducing the amount of gelatine [17], in order to obtain mixed microgels and, if necessary, study the distribution of their diameters.

The addition of alginate appears to cause a destabilization of the microgels during washing. With 1% wt alginate (Figure 15A), microparticles are indeed present, but they are trapped within a continuum of bulk hydrogel. This lack of freedom disqualifies this sample from being classified as microgels [18]. Increasing the alginate content further reduces the presence of these particles and results in the observation of what appears to be a bulk hydrogel (Figure 15B, C, D). Microgels formed from Milli-Q® water, alginate, and gelatine therefore exhibit instability, likely leading to their coalescence.

At the chemical level, unlike pure gelatine, which forms a stable hydrogel network through hydrogen bonding and physical crosslinking (helix formation) during cooling, the introduction of alginate may disrupt this process [26]. Alginate, a polyanionic polysaccharide, interacts with gelatine primarily through electrostatic and steric effects, which can weaken the gelatine network. In a pure gelatine system, the polymer chains can aggregate and form triple helices during cooling, creating a network that stabilizes the microgels. However, in a gelatine-alginate mixture, the negatively charged carboxyl groups on alginate chains can interact electrostatically with the positively charged amino groups on gelatine. These interactions reduce the availability of gelatine chains for triple-helix formation, compromising the integrity of the gelatine network.

[17] Constant total polymer mass (8 wt%).
[18] Independent particles, thus imparting a fluid-like character.

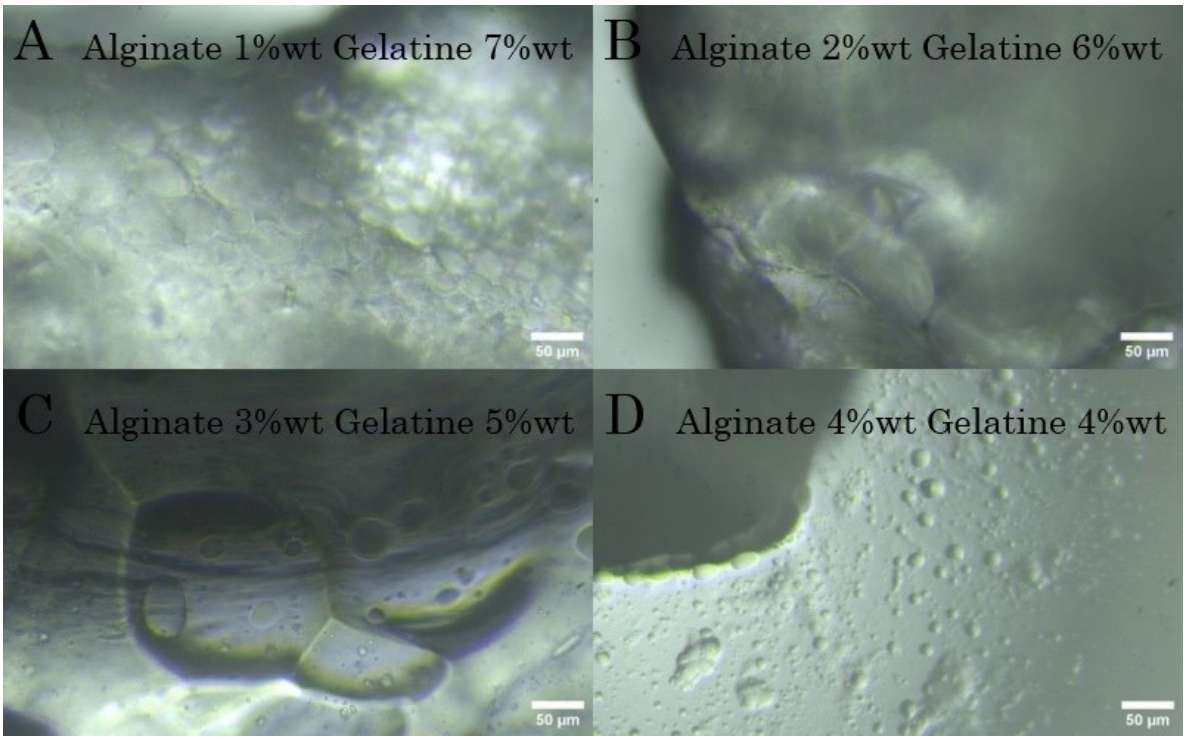

Figure 15: Microscopic images after PBS washing of samples composed of: (A) 1% wt alginate and 7% wt gelatine. (B) 2% wt alginate and 6% wt gelatine. (C) 3% wt alginate and 5% wt gelatine. (D) 4% wt alginate and 4% wt gelatine.

A chemical crosslinking of alginate was therefore attempted by adding stoichiometric proportions[19]of Ca^{2+} ions (before, during, and after the emulsification) to form "egg-box" conformations of alginate (Figure 16). The results obtained were similar to those shown in the previous figure: no microgels.

Faced with this, another protocol was tested, although it was ultimately not retained due to its lack of reproducibility and the operational burden on the operator. This involved revisiting a protocol previously used by the laboratory to synthesize GelMA microgels, where the use of a vortex at room temperature replaced centrifugation at 4°C. The results indicated an intermediate behaviour between hydrogels and microgels, regardless of the modulation in alginate and gelatine. Microscopic observations (Figure 17) and sample filtration confirmed the presence of micrometric hydrogel particles trapped in a hydrogel continuum.

The following progress was achieved thus far.

[19] The amount of ions was subsequently adjusted, with the same results.

Gelatine alone in Milli-Q® water successfully forms stable microgels. However, the incorporation of alginate destabilizes the system, leading to the formation of a continuous hydrogel matrix following the washing process. Attempts to chemically crosslink the alginate using Ca^{2+} ions proved unsuccessful. The source of this instability appears to be twofold: alginate not only induces destabilization of the gelatine network but also exhibits inherent instability. To address these challenges, a strategy involving the physical crosslinking of the entire system was investigated. This approach involved transforming gelatine into GelMA and alginate into AlMA, with the objective of stabilizing the microgels.

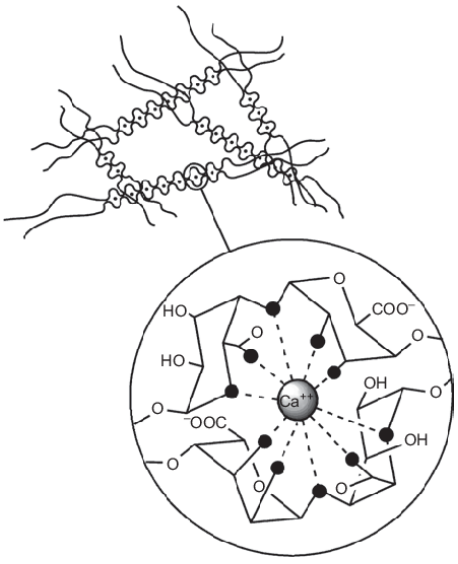

Figure 16 : Alginate's "egg-box" conformation representation. From Selimoglu *et al.*, Critical reviews in biotechnology, 2010.

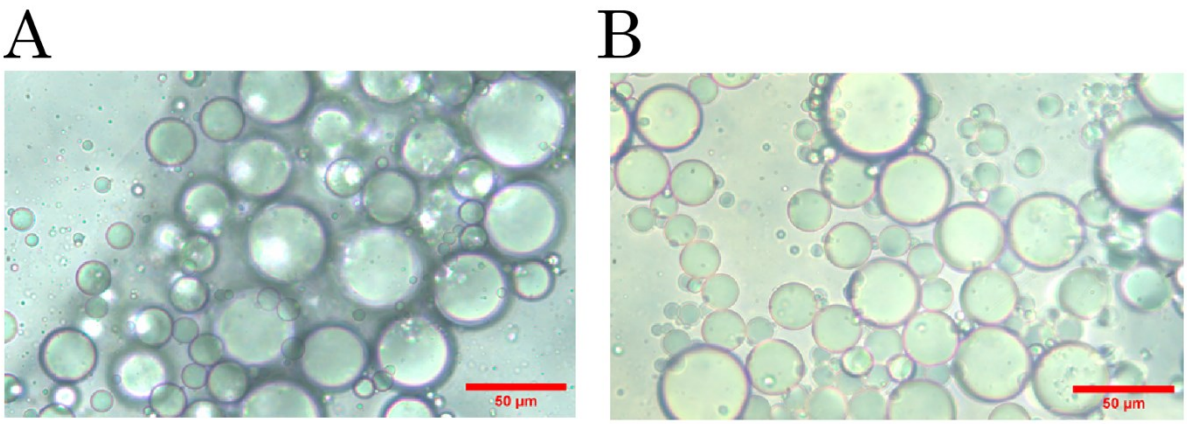

Figure 17: Microscopic images obtained from micro/hydrogel samples composed of (A) 3% wt alginate and 5% wt gelatine, and (B) 6% wt alginate and 2% wt gelatine. PBS washings were made here.

IV.4. Obtaining crosslinked particles composed of GelMA and AlMA

The instability caused by alginate, as previously described, explains the intention to achieve physical crosslinking of the system using GelMA and AlMA (NMR spectrum provided in Appendix A2). Consequently, it was logical to synthesize hydrogels analogous to those that successfully yielded gelatine microgels (IV.2), *i.e.* using Milli-Q ®water as a solvent. Following emulsification, ultraviolet crosslinking, and washing steps, the resulting products were bulk hydrogels rather than microgels (Figure 18). The particles formed during emulsification therefore underwent coalescence, this destabilization suggests that AlMA did not undergo crosslinking and may have even interfered with the crosslinking of GelMA.. Considering this, the irradiation time was adjusted; however, no improvement was achieved.

At this point, on an empirical basis, the solvent was switched to PBS. The results presented in Figure 19 differ from those obtained using Milli-Q® water as the hydrogel solvent, with particles now being observable.

Using PBS instead of Milli-Q® water as a solvent for the hydrogel precursors seems to enhance the stability of the hydrogel network during the formation of microgels. This trend was observed in the study conducted by Chen *et al.,* which demonstrated that GelMA hydrogels synthesized in PBS contain more triple-helix conformations [27]. These conformations ensure stronger polymer cohesion, as illustrated during the crosslinking of gelatine or GelMA hydrogels at low temperatures [28][29].

However, it seems prudent for now not to classify the obtained particles as microgels. A difference in morphology was observed compared to the positive control composed exclusively of GelMA (Figure 20A). The latter appears more clearly circular and does not exhibit interstitial bodies. These interstitial bodies might suggest an inhibition of particle buoyancy, preventing them from interacting with one another. Whether in the presence of GelMA alone or a combination of GelMA and AlMA, the median particle diameters are approximately 15 µm (Figure 20B).

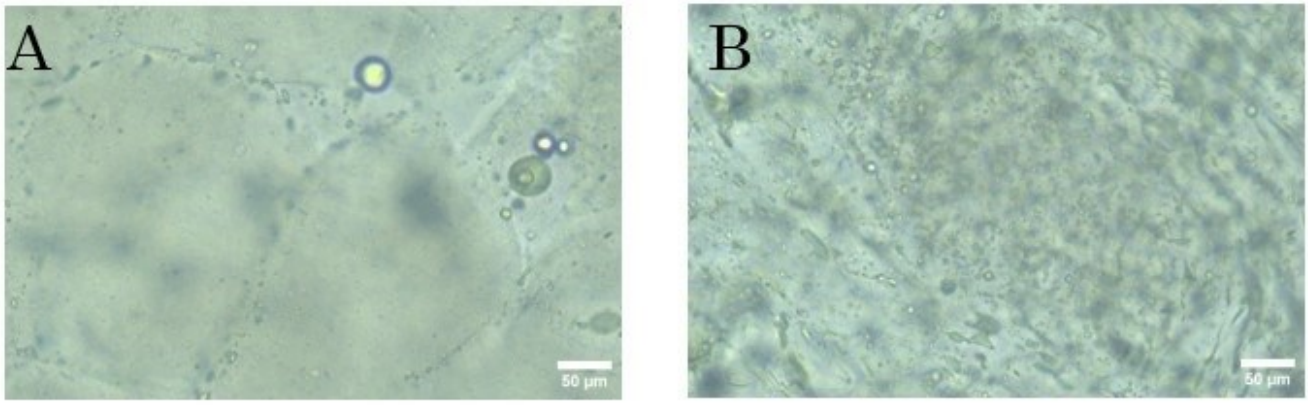

Figure 18: Microscopic pictures of destabilized microgels after PBS washing made of (A) 2% wt of AlMA and 4% wt of GelMA in Milli-Q® water (B) 4% wt of AlMA and 4% wt of GelMA in Milli-Q® water.

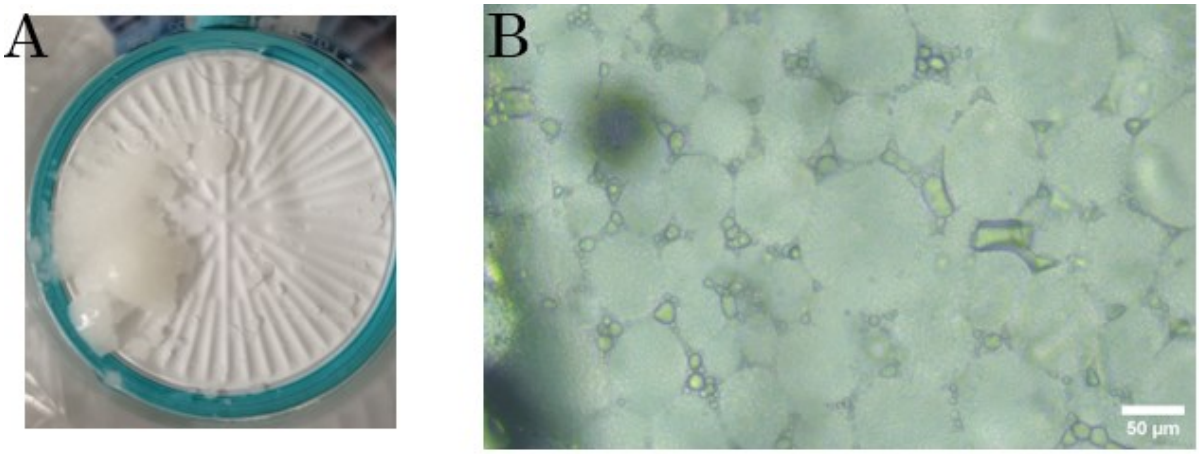

Figure 19 : Macroscopic (A) and microscopic (B) pictures of particles made of AlMA (2% wt) and GelMA (6% wt) and PBS washed in PBS.

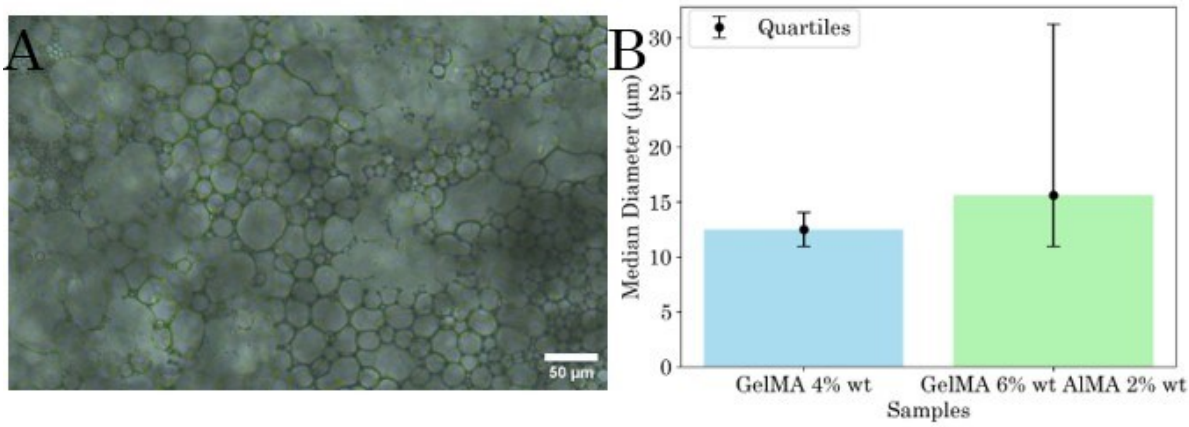

Figure 20: (A) Microscopic picture of microgels made with GelMA (4% wt) in PBS after PBS washings(B) Median diameters distribution for GelMA microgels and GelMA/AlMA particles, both made in PBS and washed using it. The measurements were performed manually.

IV.5. Rheological studies

IV.5.1. Extended viscoelastic properties provided by modulating the amount of alginate and gelatine

Apart from the microgels made of gelatine, the following rheological tests were performed on the intermediate samples between microgels and hydrogels presented in the following section. These samples were the closest results to microgels composed of alginate and gelatine at the time these rheological studies were conducted. Amplitude sweep tests were conducted with the aim of establishing an initial state of the rheological behaviour of the samples.Rigidity, viscosity, and elasticity, as indicated by the values of the two moduli, increase with the percentage of alginate (Figure 21). The viscoelastic character, illustrated by the gap between the loss and storage moduli (balance), remains constant regardless of the chemical composition. All samples exhibit a dominant elastic behaviour at low oscillations (G'>G") before transitioning to a dominant viscous behaviour (G'<G") around the same oscillation strain value (10^2%). The microgel composed of gelatine (8%wt) exhibits extreme softness compared to gelatine hydrogels with a higher concentration in gelatine (10%wt) [30]. The difference in storage modulus is indeed striking, with 10^{-3} Pa for the gelatine microgel studied here, compared to 10^2 Pa for the gelatine hydrogels investigated by Kokol *et al.* , highlighting the interest in microgels compared to the traditional hydrogels.

The results obtained are promising for the potential use of such materials as direct bio-inks *e.g.* for tissue regeneration. The balance between alginate and gelatine allows a storage modulus tuning from 10^{-3} to 10^{4} Pa, thereby exhibiting rheological properties similar to those of diverse human tissues [31].

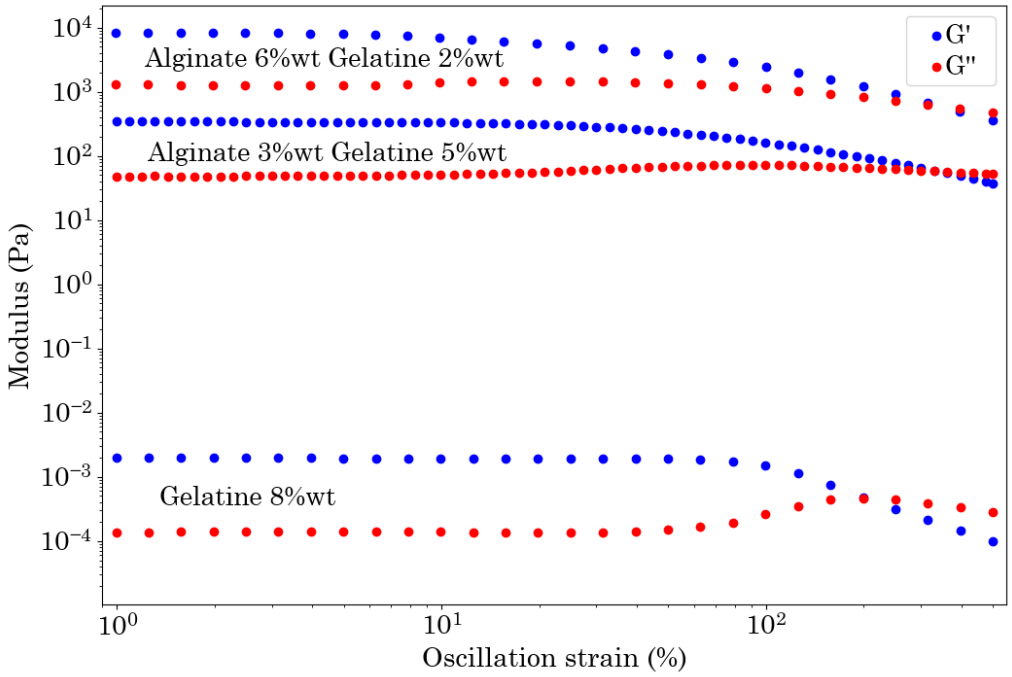

Figure 21: Extracts from amplitude sweep tests on microgels composed of gelatine and on samples identified as intermediate between microgels and hydrogels composed of alginate and gelatine. All samples were washed with PBS. G' stands for the storage modulus, and G'' for the loss modulus.

IV.5.2. A shear thinning indicating printability

The rheological characterization of the samples conducted in the previous section highlighted behaviour next to that of human tissues. However, in this context, it is essential to determine whether these gels are printable. This was investigated through flow sweep tests (Figure 22).

There is a decrease in viscosity when a perpendicular force is applied, regardless of the chemical composition of the samples. This feature, known as shear thinning [9], is crucial for the use of micro/hydrogels both as direct ink and support baths. In the first case, this allows the gels to be fluid when pushed by the printing needle, enabling them to flow freely. Once the printing pressure is removed, the gels become more viscous, which helps

preserve the structure of the printed models. Additionally, the high rigidity of the samples composed of alginate appears to make them the best candidates for 3D printing, where each layer must support the weight of the one above it. Regarding the support bath technique, a sacrificial ink is used to describe patterns within a bath made of hydro/microgels. Once removed by temperature difference, the empty tunnels created can then house a cell culture. Due to the shear thinning, the force exerted by the sacrificial ink allows for the fluidization of the micro/hydrogels, providing the necessary freedom for the 3D printing of sacrificial structures (Figure 23). A higher viscosity, $4 \cdot 10^2$ Pa·s without shear rate and $4 \cdot 10^1$ Pa·s with a shear rate of 10^2 s^{-1}, however, seems to allow for better printability [32].

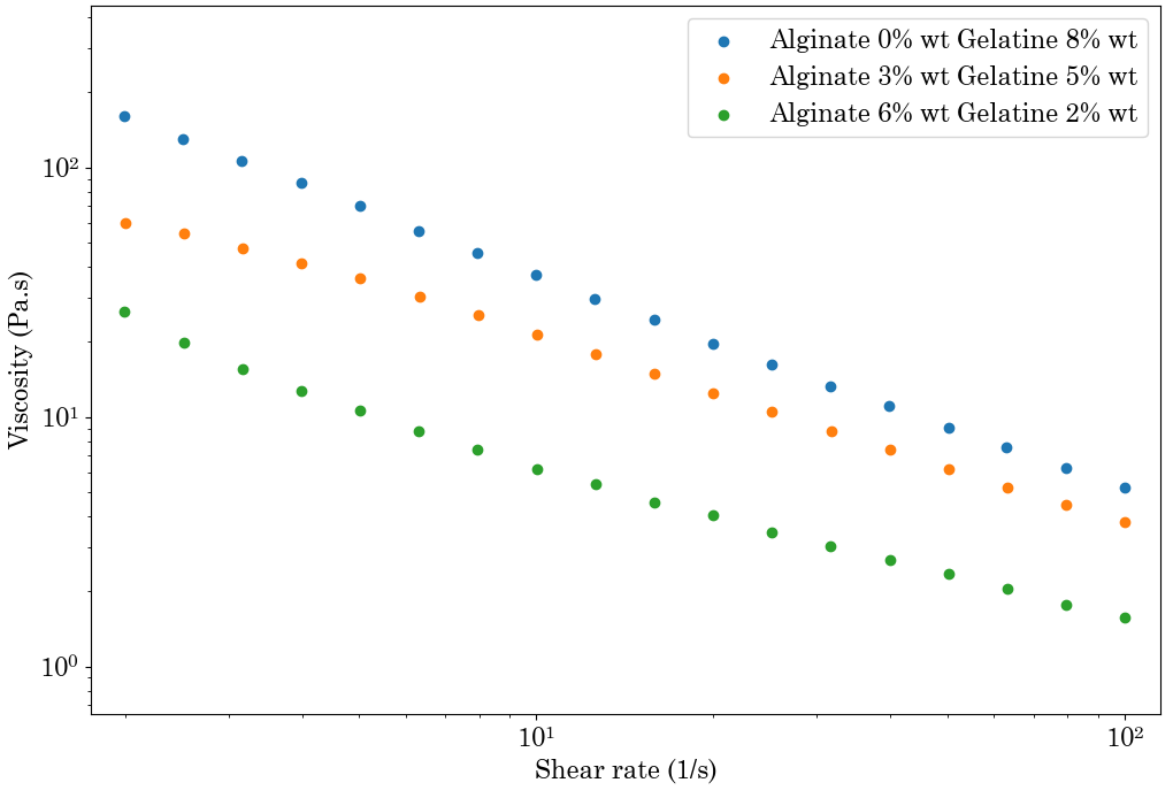

Figure 22: Extracts from flow sweep tests on micro/hydrogels washed with acetone after rehydration in PBS.

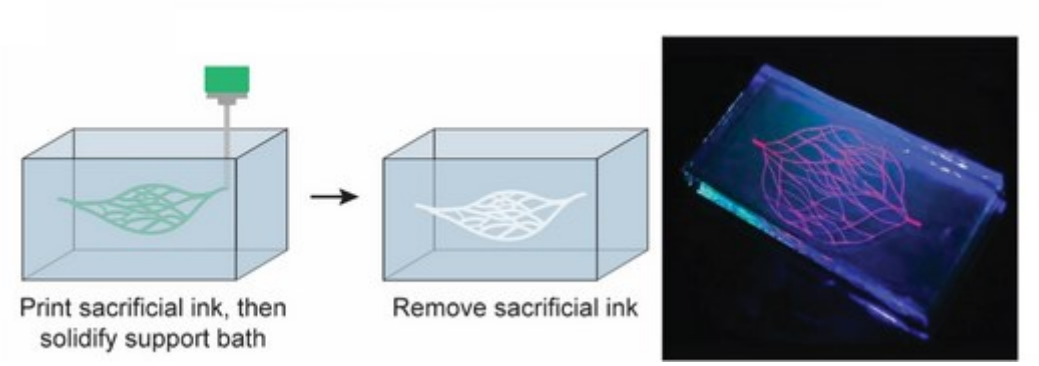

Figure 23: Illustration of a sacrificial ink printed into a support bath. From Brunel LG *et al.,* Biofabrication, 2022.

V. Conclusion

The objectives of this study were (i) to establish a protocol for obtaining microgels composed of gelatine and alginate, (ii) to characterize them in terms of diameter distribution with an arbitrary target set at 5 µm, and (iii) to study their rheology.

Microgels composed of gelatine were obtained, however this was not the case for mixtures of alginate and gelatine. The need for physical crosslinking of the polymers thus became necessary, leading to the observation of the first micrometric hydrogel particles of GelMA and AlMA. Interestingly, these particles were obtained when the hydrogels were prepared in PBS, whereas this was not the case in the presence of Milli-Q® water. Their diameters, like those of the gelatine and GelMA microgels obtained, range between 20 and 30 µm. Preliminary rheological studies indicate a wide modulation of the viscoelastic properties of the products depending on their alginate and gelatine composition.

The protocol leading to the production of AlMA and GelMA particles still needs to be improved in order to develop them into fully-fledged microgels. One approach could be to adjust the UV exposure time to achieve stronger polymer crosslinking. The production of smaller particles during emulsification could be related to the use of a finer needle or increased agitation. The computational tool also needs to be enhanced to enable the analysis of particle size distribution throughout the synthesis process. Beyond the previously mentioned Hough transform, machine learning also appears to be an interesting option. Finally, rheological tests involving exclusively microgels should be conducted to confirm the broad viscoelastic properties that alginate/gelatine systems seem to promise. Similarly, the study of printability requires additional tests, *e.g.*, shear stress vs. shear strain rate.

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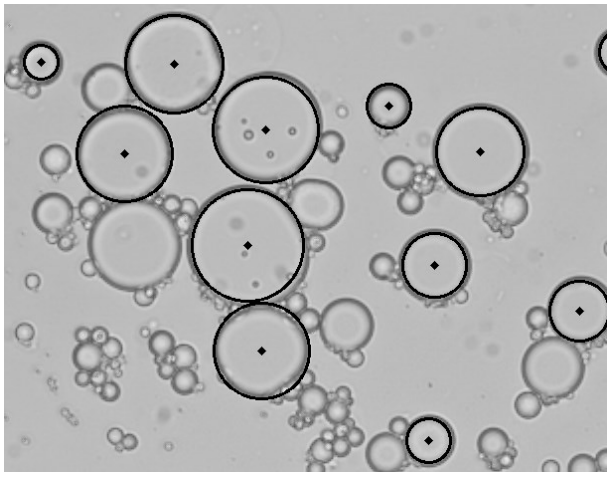
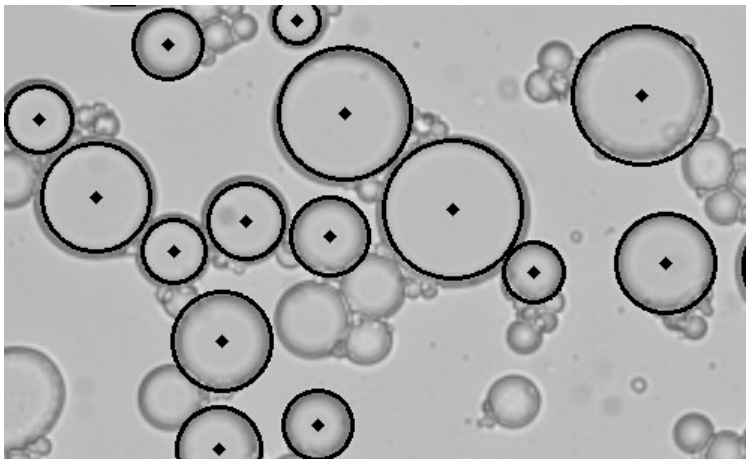
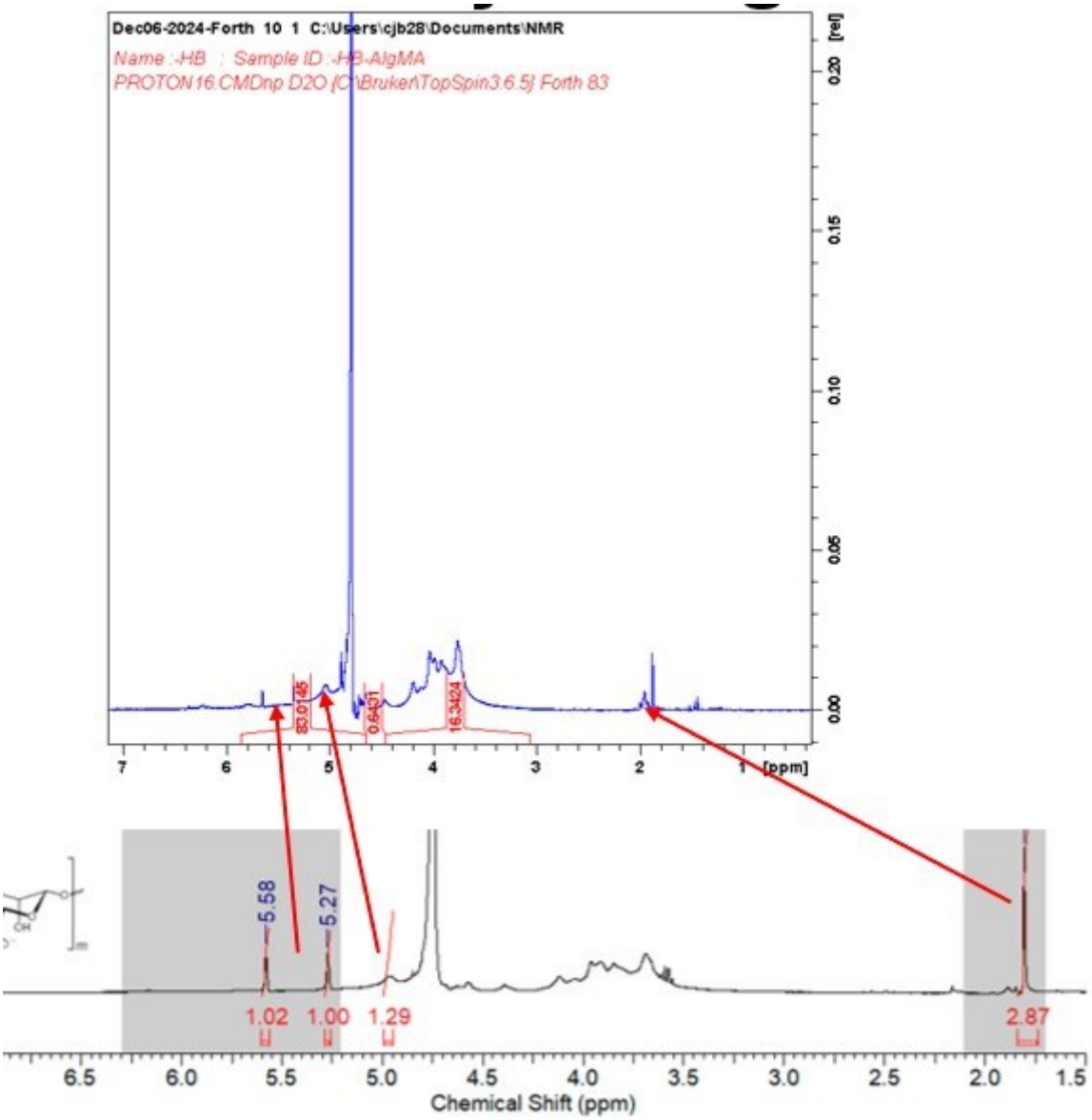
Appendix





Appendix A1: Examples of emulsified particle detections provided by the implemented code using the Hough Transform.



Appendix A2: NMR spectrum of the product obtained after AlMA synthesis (top) and comparison with the product obtained by Araiza-Verduzco et al., Materials, 2020 (bottom).

Abstracts

Exploration de méthodes de synthèses de microgels composés d'alginate et de gélatine

Les microgels, agencement de microsphères d'hydrogels injectable et poreux, constituent des matériaux d'avenir dans le traitement des blessures et la régénération des tissues. Le premier but de cette étude a été de parvenir à synthétiser des microgels composés d'alginate et de gélatine, reconnus pour leurs propriétés gélifiante et biocompatible. Après utilisation de la technique dite de *batch-emulsion* et des lavages à l'acétone ou au *phosphate-buffered-saline* (PBS), des microgels de gélatine ont été synthétisés avec succès. Cela n'a pas été le cas en présence d'alginate, ce qui a nécessité la transformation des composants en gélatine méthacrylate (GelMA) et alginate méthacrylate (AlMA) afin d'entrainer une réticulation des polymères permettant aux particules de gagner en stabilité. Une esquisse de microgels a été ainsi obtenue mais cela semble dépendre du solvant constitutif des hydrogels, à la base de la synthèse. En effet, des particules ont été discernables quand le PBS jouait ce rôle, ce qui n'a pas été le cas en présence d'eau Milli-Q®. Les études rhéologiques ont fait état d'un large domaine de propriétés viscoélastiques atteignables par modulation de la quantité d'alginate et de gélatine. Une diminution de la viscosité en fonction du taux de cisaillement a aussi été observée, indépendamment de la composition chimique des échantillons.

Exploring synthesis methods of alginate/gelatine microgels

Microgels, an arrangement of injectable and porous hydrogel microspheres, represent promising materials for wound treatment and tissue regeneration. The primary goal of this study was to synthesize microgels composed of alginate and gelatine, known for their gelling and biocompatible properties. Using the batch-emulsion technique followed by washes with acetone or phosphate-buffered saline (PBS), gelatine microgels were successfully synthesized. This was not the case with alginate, which required the transformation of the components into gelatine methacryloyl (GelMA) and alginate methacrylate (AlMA) to induce polymer crosslinking, thereby enhancing particle stability. A preliminary formation of microgels was achieved, but this seemed to depend on the

solvent used in the primary hydrogel synthesis . Indeed, particles were distinguishable when PBS served as the solvent, whereas no such particles were observed in the presence of Milli-Q® water. Rheological studies revealed a wide range of viscoelastic properties achievable by modulating the amounts of alginate and gelatine. A decrease in viscosity with increasing shear rate was also observed, regardless of the chemical composition of the samples.