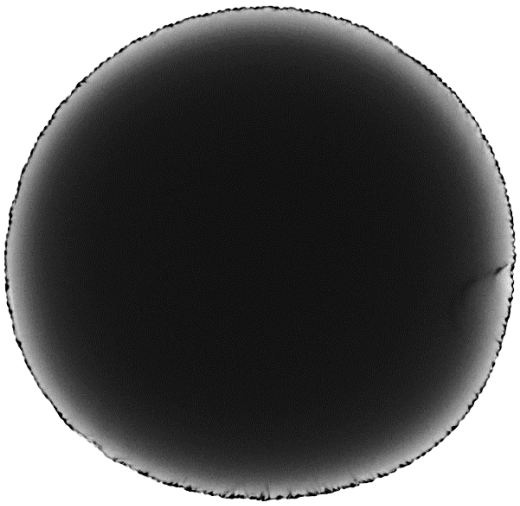
IMPACT OF SURROUNDING MEDIUMS ON THE CHEMICAL STABILITY OF IRON-LOADED ALGINATE BEADS

BACHELOR'S ASSIGNMENT BIOMEDICAL ENGINEERING



EXAMINATION COMMITTEE

Supervisor: Dr. Ir. Jeroen Rouwkema

Daily supervisor: Mira El Akkawi

External board member: Dr. Ir. Constantinos Goulas

Max Rietveld

s2398001

02-02-2024

UNIVERSITY OF TWENTE.

ABSTRACT

Alginate is a promising biomaterial in tissue engineering due to its high porosity, modification possibility, and biocompatibility. This study focuses on the influence of the surrounding medium in which the alginate and iron-loaded alginate beads are incubated. The end goal is to produce alginate beads designed for local mechanical stimulation, through their magnetic properties. The study centers on the morphological behavior and mechanical properties of both alginate and iron-loaded alginate beads with bead sizes ranging from 2.3 to 2.8 mm. Three distinct mediums and combinations of them were employed in a series of experiments, including sphericity and swelling assessments conducted over 5-7 days using EVOS microscopy and ImageJ software. Additionally, a rheometer was employed to measure compressive stiffness in a mechanical test. Results indicated that medium selection did not significantly impact sphericity over time. However, it did influence the swelling percentage, leading to chemical alterations in the alginate compounds. Beads in the cell culture medium exhibited rapid initial swelling, followed by a small reduction in size over subsequent days. In mechanical tests, these beads demonstrated lower strength compared to those induced in 0.02M $CaCl_2$, which exhibited a decrease in swelling percentage caused by an increased crosslinking density, and consequently a higher compressive stiffness. This study provides valuable insights into the complex interplay between surrounding mediums, swelling behavior, and mechanical properties of alginate and iron-loaded alginate beads, offering essential considerations for their application in tissue engineering and local mechanical stimulation.

SAMENVATTING

Alginaat is een veelbelovend biomateriaal in tissue engineering vanwege de hoge porositeit, mogelijkheid tot modificatie en biocompatibiliteit. Dit onderzoek richt zich op de invloed van het omringende medium waarin alginaat- en magnetische alginaat beads worden geïncubeerd. Het uiteindelijke doel is het produceren van alginaat beads die ontworpen zijn om lokale mechanische stimulatie te induceren door hun magnetische eigenschappen. Het onderzoek concentreert zich op het morfologisch gedrag en de mechanische eigenschappen van zowel de alginaat- als magnetische alginaat beads, waarbij de beads variëren van grootte tussen 2,3 tot 2,8 mm. Drie verschillende media en combinaties ervan zijn gebruikt in een aantal experimenten, waaronder sfericiteits en zwellingstests die gedurende 5-7 dagen werden uitgevoerd met behulp van een EVOS-microscoop en de ImageJ-software. Daarnaast werd er een rheometer gebruikt om de compressiestijfheid te meten. De resultaten toonden aan dat de mediumselectie de sfericiteit in de loop van de tijd niet significant beïnvloedde. Het beïnvloedde echter wel de mate van zwelling, wat leidde tot chemische veranderingen in de alginaatstructuur. Beads in celkweekmedium vertoonden snelle initiële zwelling, gevolgd door een geleidelijke afname van grootte in de daaropvolgende dagen. In de mechanische tests vertoonden deze beads een lage compressiestijfheid in vergelijking met de beads in 0,02M $CaCl_2$, die juist een afname in zwelling vertoonden. Dit is te verklaren door de verhoogde crosslink-dichtheid met als gevolg daarvan een hogere compressiestijfheid. Dit onderzoek biedt waardevolle inzichten in de wisselwerking tussen omringende media, zwelgedrag en mechanische eigenschappen van alginaat- en magnetische alginaat beads.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 ALGINATE IN BIOMEDICAL ENGINEERING

Biomaterials are of great value in today's world of biomedical engineering. Biomaterials are used as smart and regenerative solutions for treating diseases or injuries[1]. A biomaterial is quantified as any surface, matter, or construct that can interact with a biological system[2]. Biomaterials should therefore meet several properties to prevent failure and bodily rejection. Some examples of these properties are the acceptance of the material as mentioned before, nontoxic and noncarcinogenic, chemically stable, mechanical properties that meet the purpose of the treatment and the accessibility of the material for wide use[3]. There are many forms of biomaterials and the purpose for which they are used is still expanding. Forms of biomaterials can be natural occurring materials, plastics, living organisms, ceramics or metals[1].

One of the purposes of biomaterials lies in tissue engineering. Tissue engineering is an upcoming field of regenerative medicine. Since donor materials are still scarce, a new approach to medicine is the outcome to help patients on endless waiting lists. In tissue engineering, biomaterials are used to replace non-functional tissues and mimic the functions, both biological and mechanical, of the replaced tissue[4].

In tissue engineering, mechanical stimulation can be used as a means to improve and induce tissue functionality.[5] The regulation of tissue formation, cell activity and organic functions are highly influenced by chemical cues. Growth factors, transcription factors and cytokines are signals that play major roles in cell differentiation, migration and proliferation throughout the body[6]. Next to the chemical cues, mechanical stimulation can also contribute to the way cells behave and develop. Mechanical cues, together with the surrounding tissue, the extracellular matrix (ECM) and hormones are important for tissue formation and behavior[7]. An example of the interaction of these factors is shown in Figure 1.

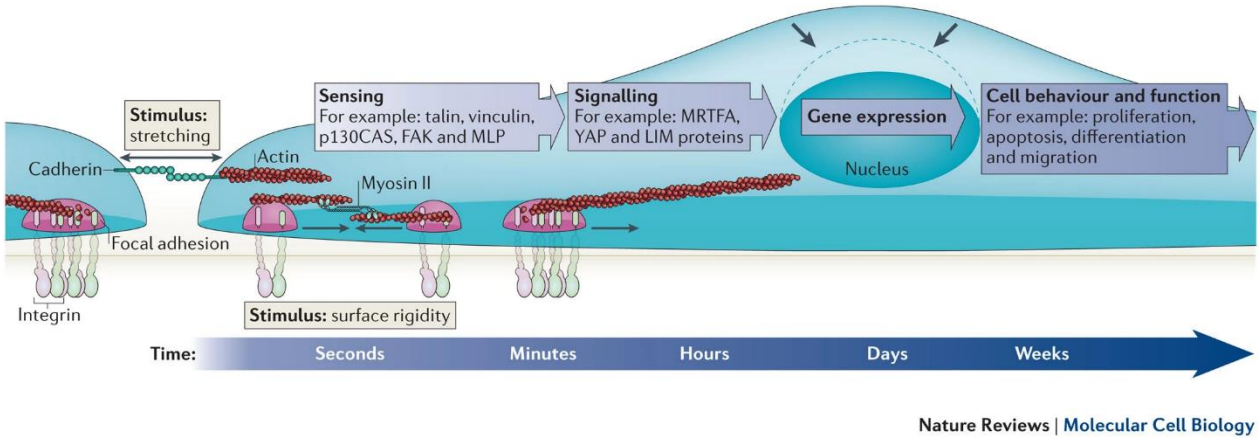


FIGURE 1: THE INTERACTION OF FACTORS INFLUENCING CELL BEHAVIOR AND FUNCTIONALITY[7]

In this study, alginate and iron-loaded alginate beads are used to test the influence of the surrounding medium on the behavior and mechanical stiffness of the alginate beads. The intended goal of producing iron-loaded alginate is to induce magneto-mechanical stimulation in tissues.

Alginate is an anionic polymer found in nature and mostly comes from brown seaweed. The polymer is blocked and built out of two copolymers, β-D-mannuronate (M) and α-L-guluronate (G). These copolymers occur with three different blocks, full M blocks, full G blocks and alternating M/G blocks[8]. The ratio between the two copolymers varies, based on the source it is obtained from[9]. To make alginate usable for medicine and tissue engineering, the polymer has to be purified to lose all possible natural compounds that can cause an immunogenic response in a patient. As alginate itself is biocompatible, its natural occurrence makes it possible for various kinds of contaminations to come along[8,10]. Iron-loaded beads should interact with surrounding tissues to stimulate them mechanically. Alginate is not bioactive, so cell-alginate interaction needs to be promoted by ligands or by crosslinking alginate with other cell adhesive compounds[8,11].

Alginate is widely used in the biomedical world. In the pharmaceutical world alginate is used as a carrier for the delivery of drugs and proteins. As alginate has high porosity properties, it can diffuse chemicals quickly through the body[8,11]. Cell culture possibilities are on the rise for alginate structures. As mentioned above, the incapability of alginate to interact with mammalian tissue makes it necessary to modify the alginate compounds before they can be used in cell culture and tissue engineering. This is also an opportunity, as alginate can serve as an empty scaffold to modify the structure to the needs of the study. Due to its high porosity, possibilities for modification and biocompatibility, alginate is a promising biomaterial in tissue engineering. Alginate is already used e.g. bone regeneration, the promotion of proteins to induce blood vessel formation and cartilage regeneration[8].

1.2 CROSSLINKING THE ALGINATE STRUCTURE

For the formation of alginate beads, a crosslinking solution is used to form spherical and chemically stable beads. When the alginate solution is extruded from the syringe into the crosslinking solution, 0.2M $CaCl_2$, the spheres are formed. This section covers the mechanism behind the bead formation.

The alginate solutions used in this study are aqueous and anionic. To obtain the beads, this aqueous solution needs to be cross-linked. Crosslinking will connect the M/G block polymers so the alginate polymer can form. This will, in combination with the impact of the solution on the alginate beads, produce spherical beads. Figure 2 shows the M and G blocks with the anionic carboxylate group and the three ways the block polymer can be formed[8].

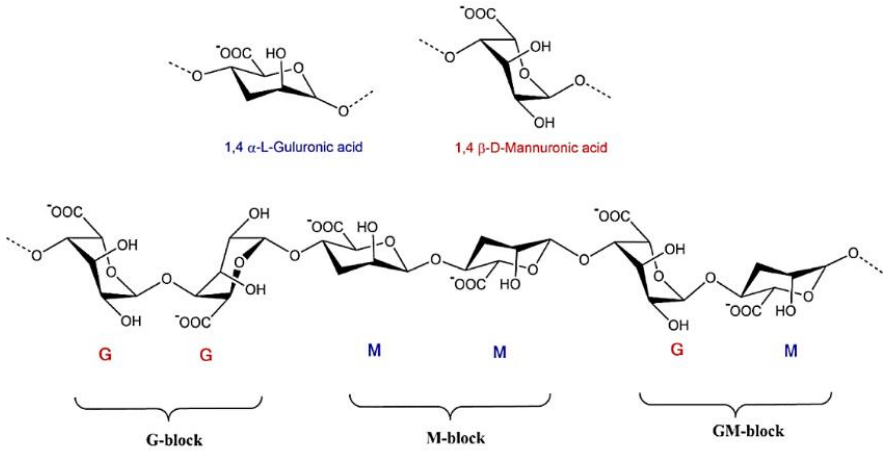


FIGURE 2: G AND M ALGINATE BLOCKS AND THREE WAYS OF BLOCK-POLYMERIZATION OF ALGINATE [11]

The mechanism used to crosslink the alginate polymers is ionic crosslinking with Ca^{2+} as the crosslinking ion. The calcium ion can form bonds with negatively charged groups like the anionic carboxylate groups of the alginate blocks. Figure 3 shows how the alginate blocks are linked with the Ca^{2+} ions and form a structured compound called an egg box.

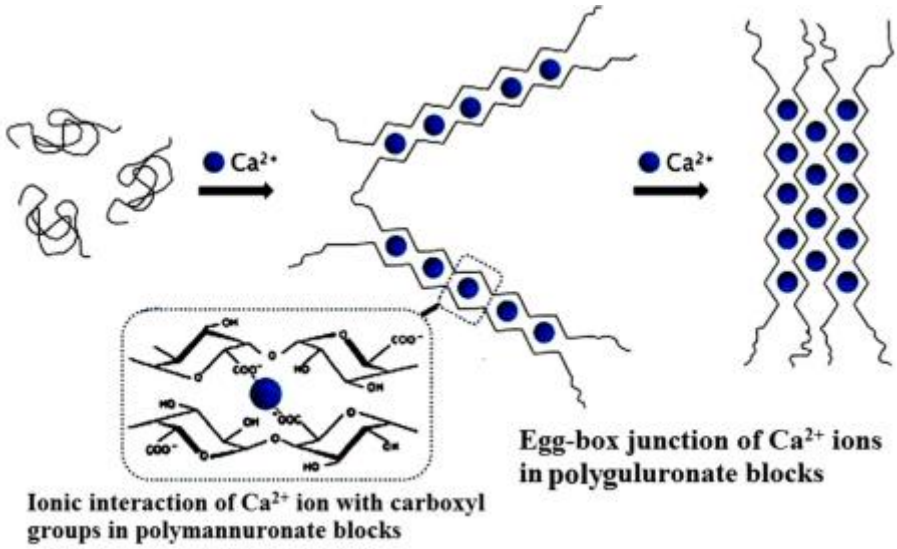


FIGURE 3: CROSSLINKING OF ALGINATE WITH CA^{2+} TO FORM A STRUCTURED COMPOUND[12]

1.3 RESEARCH GOALS

The main focus of this study is to determine the impact of the surrounding medium on the stability and functionality of iron-loaded alginate beads. The purpose of the iron-loaded alginate beads is to apply mechanical stimulation on cells and therefore the beads must be able to withstand certain conditions to be suitable for cell culturing. These conditions are examined according to the following research question: *What is the impact of the surrounding medium on the chemical stability of iron-loaded alginate beads?*

The objective involves conducting multiple experiments using various mediums, diverse concentrations of alginate, and varying weights of magnetic load. This aims to comprehensively understand how these factors collectively influence the behavior of alginate beads. Following the medium experiments, the mechanical characteristics of the alginate beads in these distinct conditions are examined using a rheometer. This analysis provides valuable insights into the mechanical behavior of the alginate beads when swelling or shrinking and surrounded by different media.

2 METHODS

2.1 MATERIALS

The alginate beads were produced with sodium alginate. To crosslink the solution calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) was used. All Milli-Q was tapped from the Merck Millipore Milli-Q EQ 7000. To fabricate the beads, the Harvard Apparatus PHD Ultra syringe pump was used. The syringe pump used two 60 mL syringes of BD Plastipak. Attached to the syringe was a 27G or 23G nozzle from Needlez. To filter the alginate or $CaCl_2$ solutions, 32 mm acrodisc syringe filters with 0.45 µm supor membranes from Pall were used. After production, all used well-plates (Greiner) or petri dishes were non-tissue culture material. The used strainers for the distribution of the beads were 100µm Easystrainers from Greiner. The mediums used for incubation were α-MEM and PBS, both from Thermo Fischer Scientific. All imaging was done with the Thermo Fischer Scientific EVOS microscope. The compression tests were executed with the Discovery HR20 rheometer from TA Instruments.

2.2 EXPERIMENTAL SET-UP

2.2.1 PRODUCING OF THE ALGINATE BEADS

A syringe pump was used to produce all beads. The syringe with the alginate or iron-loaded alginate solution is added and fixed to reduce changes in set-up. An empty syringe was added to the pump as a counterbalance to the alginate/alginate-iron solution. The flow out of the syringe was set at 150 mL/h. This outflow is intended to create beads with a diameter of around 2.8mm with a 23G 90º angle nozzle and 2.3mm with a 27G 90º angle nozzle[13]. The nozzle was attached to the syringe to drop the alginate or iron-loaded alginate solution into a mixing $CaCl_2$ + 10% ethanol crosslinking solution. The distance between the crosslinking solution and the nozzles was 3.5cm[14]. Figure 4 shows the setup of the syringe pump used to distribute the alginate and iron-loaded alginate beads.

FIGURE 4: SET-UP OF HARVARD APPARATUS PHD ULTRA SYRINGE PUMP WITH 23G NOZZLE TO PRODUCE BEADS.

The first experiment was considered as a pilot experiment. The produced batches of beads were used as a test to examine the behavior of the alginate - and iron-loaded alginate beads in different media. Where the first bead experiment took 3-5 beads per well out of the available 10, the second medium experiment only had one bead per well, which made sure that the same beads were imaged and analyzed daily. Therefore, the first medium experiment gave an insight into the behavior of the beads in the different mediums. After that, a choice of conditions was made for further investigation and broader analysis.

2.2.2 First medium experiment

In the first medium experiment, the beads were made with the 23G nozzle and placed in two 12-well plates with approximately 10 beads per well. The first well plate was used for all combinations of 20 g/L alginate beads and the second well plate for all 30 g/L alginate bead combinations. Both concentrations of beads were made in alginate form, alginate + 1% w/v iron powder and alginate + 10% w/v iron powder. Three different mediums were added to the alginate and iron-loaded alginate beads, this is shown in Table 1. All used 0.02M $CaCl_2$ as a medium was filtered with a 0.45µm filter before use, making sure the all particles were removed and the medium was clean.

Column	Medium
1	α-MEM
2	PBS
3	0.02M $CaCl_2$

Table 1: Mediums of the first experiment

Each well was filled with 2 mL of medium. Over time the beads were placed inside the 37°C incubator. The design of the two well plates is shown in Figure 5.

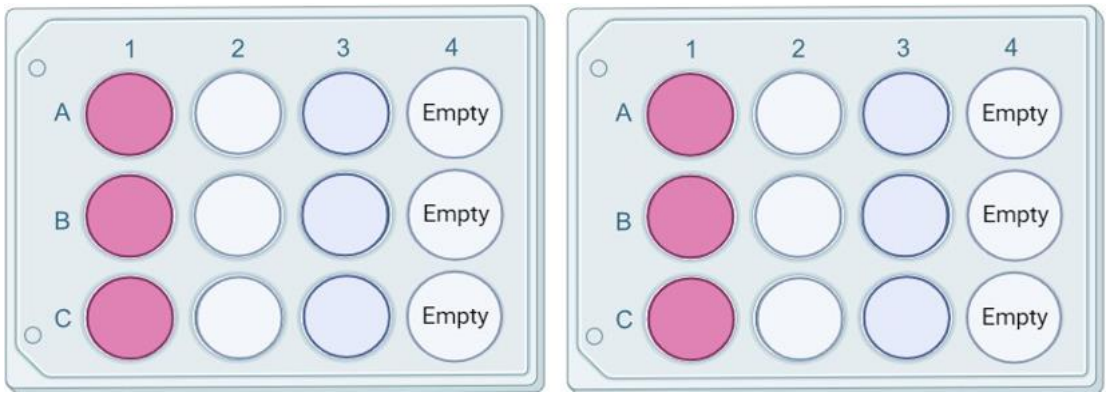


Figure 5:Left 12 wells plate with 20 g/L alginate beads. Right 12 wells plate with 30 g/L alginate beads. In botH, row A contained the alginate beads, row B the 1 w/v% iron-loaded beads, and row C the 10% w/v iron-loaded beads

2.2.3 Second medium experiment

For the second medium experiment, the beads were made with a 27G nozzle and afterward placed in two 24-well plates. This time, for faster and easier analysis, one bead was placed per well. The change of nozzle was intended to produce smaller beads: during the first experiment, not all beads did not fit the EVOS image size. The first well plate was used for the 20 g/L alginate beads and the second well plate for the 20 g/L alginate + 10% w/v iron powder beads. Three mediums and combinations of mediums were used for research on the alginate and iron-loaded alginate beads. The first medium was the cell culture medium(CM), which is α-MEM + 10% FBS + 1% penicillin/streptomycin. All six mediums are shown in Table 2.

Column	Medium
1	100% CM
2	75% CM + 25% 0.02M $CaCl_2$
3	50% CM + 50% 0.02M $CaCl_2$
4	100% 0.02M $CaCl_2$
5	50% PBS + 100% 0.02M $CaCl_2$
6	100% PBS

Table 2: Mediums of the second experiment

Each well was filled with 1 mL of medium. Over time, the beads were placed inside the 37°C incubator. The design of the two well plates is shown in Figure 6.

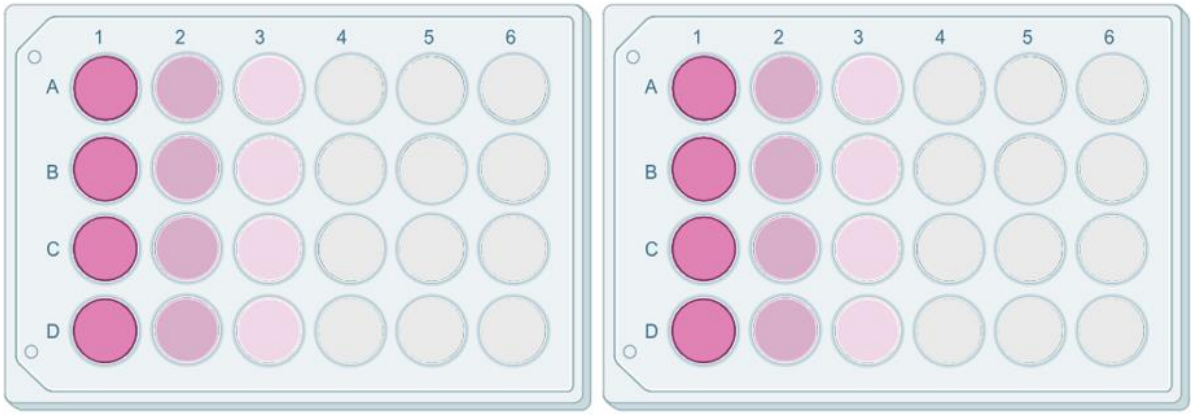


FIGURE 6: LEFT 24- WELLS PLATE IS FILLED WITH ONE 20 G/L ALGINATE BEAD PER WELL. B) RIGHT 24- WELLS PLATE FILLED WITH ONE 20 G/L ALGINATE + 10% W/V IRON POWDER BEAD PER WELL. BOTH WELL PLATES ARE IDENTICALLY FILLED WITH MEDIUM.

2.3 EXPERIMENTAL DESIGN

2.3.1 PROTOCOL PRODUCING ALGINATE BEADS AND IRON-LOADED ALGINATE BEADS

To produce the beads for the different medium experiments, a step-wise protocol is to be followed. This protocol goes through all processes necessary for the production, washing and sterilization of the alginate beads. The sterilization process is set at one hour. 70% ethanol needs 30 minutes to kill all living organisms and the extra 30 minutes are necessary for the 70% ethanol to replace the 0.2M $CaCl_2$ + 10% ethanol or Milli-Q inside the beads. After sterilization, the beads are ready to use for experiments.

2.3.1.1 ALGINATE AND IRON SOLUTION

- Weigh the alginate and iron powder necessary
- Add the alginate to mixing milli-Q in clean glasswork
- Add the alginate solution through a 0.45µm filter in the labeled 50 mL tube
- If the alginate solution was pre-made also filter through a 0.45µm filter before production or the addition of iron
- If iron-loaded, add the iron powder to the tube. Don't forget to mix by hand or roller mixer if not for direct use.

2.3.1.2 PRODUCING CROSSLINKING SOLUTION

- Weigh the $CaCl_2$ crystals necessary for a 0.2M $CaCl_2$ + 10% ethanol solution
- Calculate the amount of 70% ethanol necessary for the 0.2M $CaCl_2$ + 10% ethanol solution
- Calculate the amount of milli-Q necessary for the crosslinking solution[15]
- Put together in a clean labeled glass or plastic bottle with a cap and start stirring
- Stir till the $CaCl_2$ crystals are fully dissolved

2.3.1.3 PRODUCING IRON-LOADED ALGINATE BEADS

- First add the counterbalance (empty syringe) to the syringe pump
- Open the new syringe
- Add the combi stopper to prevent leakage of fluid
- Pour the alginate or iron-loaded alginate solution inside the syringe.
- Then loosen the combi stopper while holding the pump to prevent opening the syringe by pressure difference

- Add the 23g 90º or 27g 90º angle nozzle to the syringe with the needle aiming downwards
- Put the tube with the crosslinking solution below the syringe at 3.5 centimeters distance from the solution to the nozzle. Always on the same distance from nozzle to solution to prevent differences in the experiments
- Start the syringe pump with the following instructions: infuse, 150ml/h
- Start shaking the tube with the crosslinking solution while beads are being formed to decrease surface tension[16]

2.3.1.4 CLEANING AND STERILIZATION OF THE IRON-LOADED ALGINATE BEADS

- First dispose the crosslinking 0.2M $CaCl_2$ + 10% ethanol solution with a pipet
- To wash the beads add Milli-Q till the beads are fully covered in solution
- Wash the beads by gently turning the flask round
- Extract the Milli-Q and repeat the washing step another two times
- Extract the Milli-Q for sterilization
- To sterilize the beads add 70% ethanol till the beads are fully covered in fluid
- Leave the beads in 70% ethanol for at least an hour for sterilization
- If not for direct use put the beads with 70% ethanol in the 4°C fridge

2.3.1.5 ADDING BEADS TO THE MEDIUM

- First prepare the different mediums
- Put the different mediums in the thermal bath to pre-heat the media
- Extract the 70% ethanol from the flask in the LAF cabinet with the aspiration system
- Use a strainer on a waste tube to collect the beads from the flask and further dispose of the 70% ethanol
- Add the beads to the wells plate (beads with preferred sphericity =/> 95%)
- Then add the different mediums, 2mL per 12-wells plate and 1mL per 24-wells plate, to the wells
- After use always put the well plates in the 37ºC incubator

2.4 MECHANICAL STIFFNESS ANALYSIS

FIGURE 7: DISCOVERY HR20 RHEOMETER

The analysis of the mechanical stiffness was executed with a rheometer. The rheometer is capable of measuring various mechanical properties of compounds. Three different conditions with two different beads were analyzed. 20 g/L alginate beads and 20 g/L alginate + 10% w/v iron beads have been placed in 100% culture medium, 50% culture medium + 50% 0.02M filtered $CaCl_2$ and 100% 0.02M filtered $CaCl_2$. The beads have been in the mediums in the 37°C incubator for 5 days prior to the analysis. The 5-day incubation is chosen from the data of the second medium experiment, after noticing that most swelling and deformation ended before the 5th day. For each condition, four beads were analyzed. The rheometer was coded in two steps for analysis. The first step, called the pre-tension step, consists of lowering the 8mm compressing plate at a rate of 50µm/s until it touches the beads and the axial force reaches $5 * 10^{-3}$ N, the minimal force to be recorded on the rheometer. After the pre-tension step, the compression step is automatically started. The compressing speed here is 300µm/s and the compressing stops when the gap is approximately 66% of the initial diameter of the beads. As the diameters of the beads vary, the gap where the compressing stops has to be entered manually. Figure 7 shows the rheometer used in this analysis.

In this study, the axial force is studied in function of the gap. From the gap, the displacement can be computed. This data can be used in the Hertz equation that gives the relation between the force F and the displacement H. Equation (1) shows the Hertz equation[17].

$$F = \frac{4\sqrt{R}}{3}\frac{E}{1-v^2}(H)^{\frac{3}{2}} \quad \textbf{(1)}$$

In this equation, F is the axial force in (N), R the radius of the beads in (m), E the Young's Modulus in (N/m^2) or (Pa), v the Poisson ratio and H the displacement in (m). The Poisson ratio is 0.5, because the beads can be considered incompressible at the used speed[17]. The Hertz equation can now be rewritten to equation (2).

$$F = \frac{4}{2.25}\sqrt{R}E(H)^{\frac{3}{2}} \quad \textbf{(2)}$$

Eventually, the parameter wanted is the Young's Modulus, which gives the compressive stiffness of the alginate or iron-loaded alginate beads. From (2), $\frac{4}{2,25}\sqrt{R}E$ can be considered as the slope of the equation, which can be computed from the rheometer data. This slope can then be rewritten to $E = \frac{slope}{\sqrt{R}}\frac{2.25}{4}$. Now, the axial force F, the displacement H and the compressive stiffness E are known parameters that can be solved.

2.5 MORPHOLOGY ANALYSIS

For characterization and determination of the chemical stability of the (iron-loaded) alginate beads, the sphericity and swelling percentage are important factors. To determine the sphericity, the diameter of the beads needs to be calculated and for the swelling percentage, the area is the parameter to define. To determine the diameter and area of the alginate beads, the well plates or petri dishes are examined with the EVOS microscope. The EVOS microscope is used with GPF filter in transmitted condition, the objective at 2x and without diffuser.

2.5.1 BEAD SIZE AND SPHERICITY ANALYSIS

The images taken with the EVOS microscope are used to analyze the results. With the imaging software ImageJ, the diameter and area of the beads were determined in micrometers (µm). First, a scale needed to be set to convert the size parameter from pixels to micrometers. This process is shown in Figure 8A. From multiple analyses, now known is that 552 pixels is 2000µm. This scale was used in all further ImageJ analyses. After the determination of the scale, the diameters were determined. For each bead, 4 to 6 different diameters were measured and the biggest and smallest diameters were used to calculate the sphericity. For spherical-looking beads 4 diameters were measured and for more oval or other shaped beads 6 diameters were measured, because those beads have more variance between diameters. The calculation of the diameter is illustrated in Figure 8B.

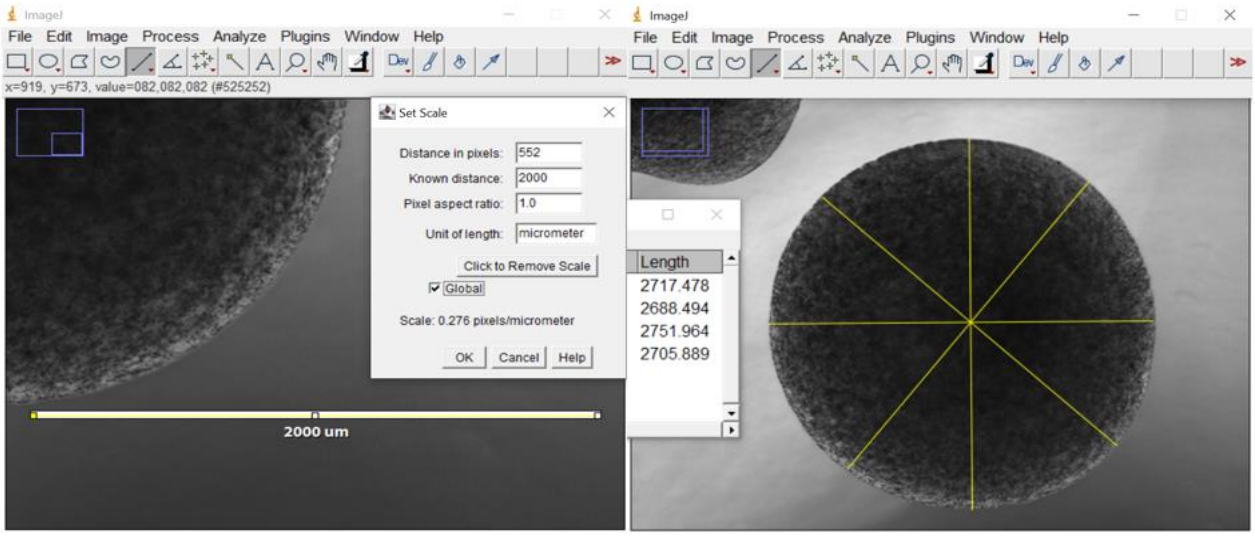


FIGURE 8: A) SETTING A SCALE IN IMAGEJ WITH EVOS IMAGE. B) DETERMINATION OF DIAMETERS WITH IMAGEJ SCALE.

To calculate the sphericity of the alginate and iron-loaded alginate beads, equation (3) was used[18]. This equation gives an outcome between 0 and 100%. Here, 100% means a perfectly round shaped beads. In this study, the outcome is factored into a percentage. If the sphericity of the bead is higher than 95%, the bead is spherical. Between $< 90\%, 95\% >$ the bead is oval or pear-shaped. When the bead reaches a lower spherical percentage, the bead is considered to be critically deformed.

$$Sphericity = \frac{2d_{min}}{d_{min} + d_{max}} * 100(\%) \quad (3)$$

2.5.2 SWELLING ANALYSIS

To determine the swelling behavior of the beads in the different conditions in all the experiments, the area was the most important parameter. The comparison of the area between different analysis moments gives the swelling percentage. The areas were determined with the software ImageJ. The same scale was used as in 2.5.1 at all times. Areas were given and calculated in μm^2. Figure 9 shows the segmentation of the alginate bead in ImageJ.

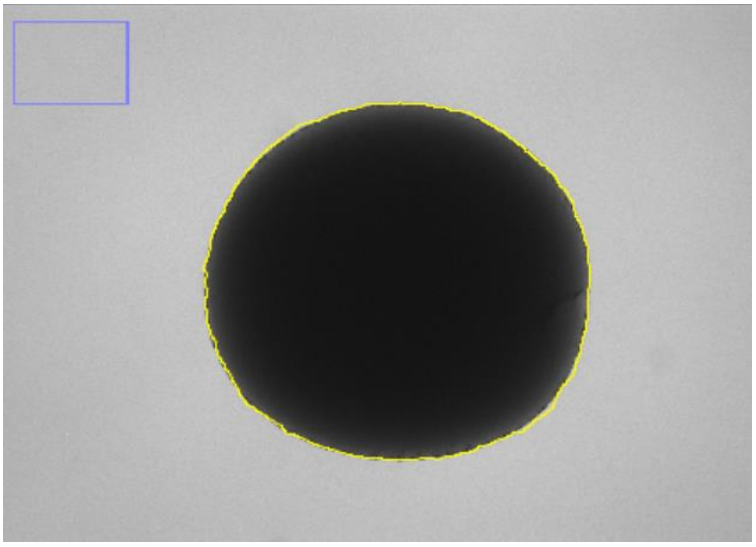


FIGURE 9: BEAD SEGMENTATION IN IMAGEJ

The swelling percentage was calculated by equation (4). In this equation area T_n is the area at the moment the swelling percentage is calculated and area T_{n-1} is the area of the prior calculated moment.

$$Swelling\ percentage\ (\%) = \frac{(Area\ T_n - Area\ T_{n-1})}{Area\ T_{n-1}} * 100\% \quad \textbf{(4)}$$

3 RESULTS

3.1 MORPHOLOGY ANALYSIS OF ALGINATE AND IRON-LOADED ALGINATE BEADS

3.1.1 FIRST MEDIUM EXPERIMENT

In the first medium experiment, 18 different conditions were studied over a period of 7 days. Alginate beads with 20 g/L and 30 g/L concentrations in pure form, 1% w/v iron-loaded and 10% w/v were examined in α-MEM, 0.02M $CaCl_2$ and PBS. From each condition, 3-5 beads were analyzed daily. The results of these 3-5 beads are the average over the 7 days, including a standard deviation over the whole calculation.

The results of the sphericity study of the first experiment show that the 10% v/w iron-loaded alginate beads were the most spherical in α-MEM. The 20 g/L alginate beads showed that the 10% w/v iron-loaded beads were pear-shaped after 7 days, while the 30 g/L alginate + 10% w/v iron-loaded beads can be considered spherical. All 1% w/v iron-loaded alginate beads in α-MEM were considered to be pear-shaped after 7 days. Next to that, the 20 g/L pure alginate beads were less spherical in α-MEM than the 30 g/L alginate beads. In PBS, most concentrations of alginate and iron resulted in spherical beads. Only the 20 g/L pure alginate beads can be considered non-spherical, while the 20 g/L 1% iron-loaded alginate beads are pear-shaped. The last medium, 0.02M $CaCl_2$, showed all 30 g/L combinations at 96% sphericity or higher and thus spherical. The pure alginate 20 g/L form and the 20 g/L 1% w/v iron-loaded alginate beads are between 90% and 95% sphericity and thus pear-shaped. The 20 g/L 10% w/v iron-loaded alginate beads have a sphericity percentage of 96,2% and are therefore spherical.

The initial size of the alginate beads in α-MEM was between 2.698 mm to 3.326 mm. Both 20 g/L and 30 g/L concentrations fluctuate between this size. For PBS the initial size was in the range of 2.651 mm to 3.442 mm. 0.02M $CaCl_2$ ranged between 2.522 mm and 2.752 mm. After 7 days PBS' size lies between 2.948 and 3.593 mm, which means that it has swollen. The swelling percentage over time of α-MEM is shown in Figure 10 and that of 0.02M $CaCl_2$ is shown in Figure 11.

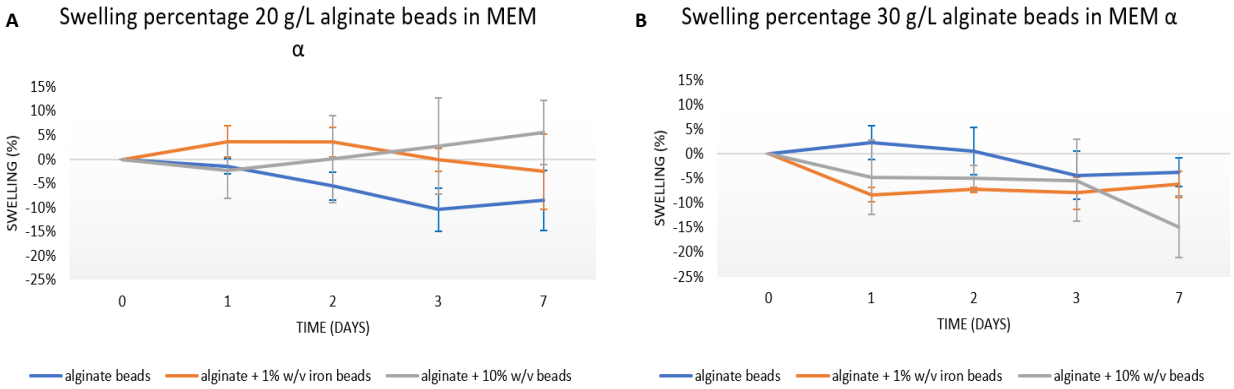


FIGURE 10: A) SWELLING PERCENTAGE OF 20 G/L ALGINATE BEADS IN ALPHA-MEM B) SWELLING PERCENTAGE OF 30 G/L ALGINATE BEADS IN ALPHA-MEM WITH THE STANDARD DEVIATION

Figure 10 shows that both pure alginate and 1% w/v iron-loaded alginate beads shrank over time with both concentrations of alginate in α-MEM. However, 10% w/v iron-loaded beads had opposite behaviors to the different concentrations of alginate.

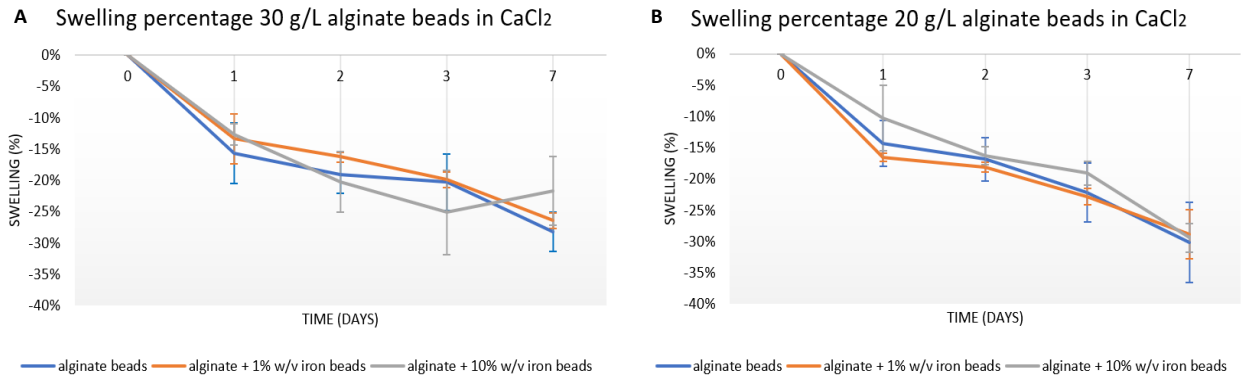


FIGURE 11: A) SWELLING PERCENTAGE OF 20 G/L ALGINATE BEADS IN 0.02M $CaCl_2$ B) SWELLING PERCENTAGE OF 30 G/L ALGINATE BEADS IN 0.02M $CaCl_2$ WITH THE STANDARD DEVIATION

Both concentrations of alginate in Figure 11 show that over time the alginate and iron-loaded alginate beads shrink in 0.02M $CaCl_2$. In all six of the conditions, the beads were 70-80% of their initial size at the start after 7 days of analysis. With the error included, both concentration and all three different beads show no significant difference in swelling percentage over time.

3.1.2 SECOND MEDIUM EXPERIMENT

The second medium experiment consists of only two different combinations of concentrations. The 20 g/L pure alginate beads and the 20 g/L 10% w/v iron-loaded alginate beads. Four wells per condition and one bead per well. The 10% w/v iron loaded showed the best sphericity of the two iron concentrations in the first medium experiment. As the difference between 30 g/L and 20 g/L is not significant, the choice is made to continue with the 20 g/L alginate concentration. The α-MEM medium is now changed to the actual culture medium (CM) α-MEM + 10% FBS + 1% pen/strep. Further, the 0.02M $CaCl_2$ and PBS solutions are still in use. The main focus is the ratio between CM and $CaCl_2$, which is investigated with 100% CM, 75% CM + 25% $CaCl_2$, 50% CM + 50% $CaCl_2$ and 100% $CaCl_2$. Next to that, a 50/50 medium of $CaCl_2$ and PBS and a 100% PBS medium are examined.

As a start, the pure PBS medium dissolved both different beads after the first 24 hours. The initial sphericity of day 0 was between 85%-98%. Figure 12 shows a 10% w/v iron-loaded alginate bead in PBS at day 0 and day 1. Noticeable is loose iron powder in the well. The initial size of the alginate beads in PBS was 2.659 mm to 3.107 mm. The range of the pure alginate beads was 2.659 to 2.731 mm and that of the alginate + 10% w/v iron alginate beads was 2.876 to 3.107 mm. As the beads dissolved after one day, there was no swelling analysis.

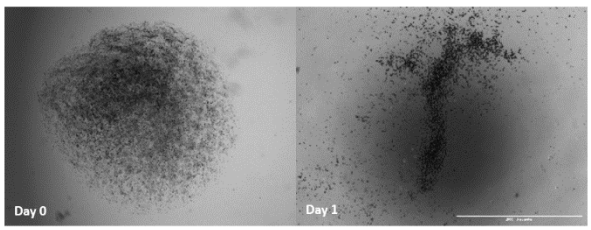


FIGURE 12: 10% W/V IRON LOADED ALGINATE BEAD IN PBS AT DAY 0 AND DAY 1. SCALEBAR IS 2000µM

The 50% PBS + 50% 0.02M $CaCl_2$ media reacted with each other, resulting in a precipitation. This did not affect the beads or analysis. The beads were very stable in sphericity from day 0 to day 7. The 20 g/L alginate beads started at 94.40% ± 0.15% and ended at 94.69% ± 0.37%. The 10% w/v iron-loaded alginate beads started at 92.21% ± 1.64% and ended at 92.09% ± 1.61% at day 7. The swelling of both conditions on day 7 was a decrease, one of 12.38% ± 3.21% for the pure alginate form and one of 12.26% ± 4.13% for the iron-loaded condition. Figure 13 shows the precipitation of day 0 and day 7.

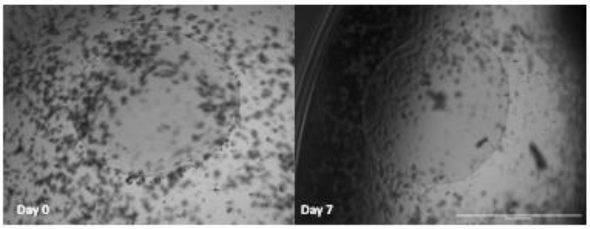


FIGURE 13: PRECIPITATION OF PBS AND $CaCl_2$ WITH 20G/L ALGINATE BEAD AT DAY 0 AND DAY 7. SCALEBAR IS 2000µM.

The 100% culture medium showed minimal difference in sphericity over 7 days. The pure alginate condition started at 92.03% ± 0.96% sphericity and ended on day 7 at 92.82% ± 0.67%. The 10% w/v iron-loaded alginate beads went from 91.91% ± 1.30% to 92.13% ± 0.74% sphericity. For the other four conditions, the results are shown in Table 3 and Table 4.

	75% CM + 25% $CaCl_2$	**50% CM + 50% $CaCl_2$**	**100% $CaCl_2$**	**50% $CaCl_2$ + 50% PBS**
Sphericity day 0 (%)	92.81	92.97	95.95	94.40
Sphericity day 7 (%)	94.03	93.38	93.65	94.69
Standard deviation day 7	1.09%	0.58%	1.35%	0.37%

TABLE 3: SPHERICITY STUDY OF FOUR DIFFERENT 20 G/L ALGINATE BEAD CONDITIONS

	75% CM + 25% $CaCl_2$	**50% CM + 50% $CaCl_2$**	**100% $CaCl_2$**	**50% $CaCl_2$ + 50% PBS**
Sphericity day 0 (%)	92.93	93.97	92.44	92.21
Sphericity day 7 (%)	92.61	92.65	92.54	92.09
Standard deviation day 7	0.46%	1.06%	1.02%	1.61%

TABLE 4: SPHERICITY STUDY OF FOUR DIFFERENT 20 G/L ALGINATE + 10% W/V IRON LOADED BEAD CONDITIONS

There were little changes in the sphericity over the 7 days in most conditions. Table 3 and Table 4 show sphericities that all lie in each other's error boundaries. Only the 20 g/L alginate bead in 0.02M $CaCl_2$ showed a change in spherical form after 7 days, from spherical to pear-shaped. The rest was all pear-shaped over 7 days' time.

The swelling analysis of the beads in CM and $CaCl_2$ media showed a decrease in size over time in all four of the conditions. The initial size of the 100% CM alginate beads was 3.039 mm – 3.216 mm. After 7 days this ranged from 2.647 mm – 2.895 mm. The 10% w/v iron-loaded alginate beads went from 2.945 mm – 3.491 mm to 3.059 mm - 3,170 mm after 7 days in the medium. On the other side, the 100% 0.02M $CaCl_2$, showed much smaller initial sizes at day 0. The pure alginate beads started at 2.188 mm – 2.248 mm and the iron-loaded alginate beads started at 2.113 mm – 2.379 mm. After 7 days in the 0.02M $CaCl_2$ medium, both conditions ranged from 1.797 mm – 1.910 mm. Figure 14 shows the swelling percentages of all four conditions over the 7 days in the medium.

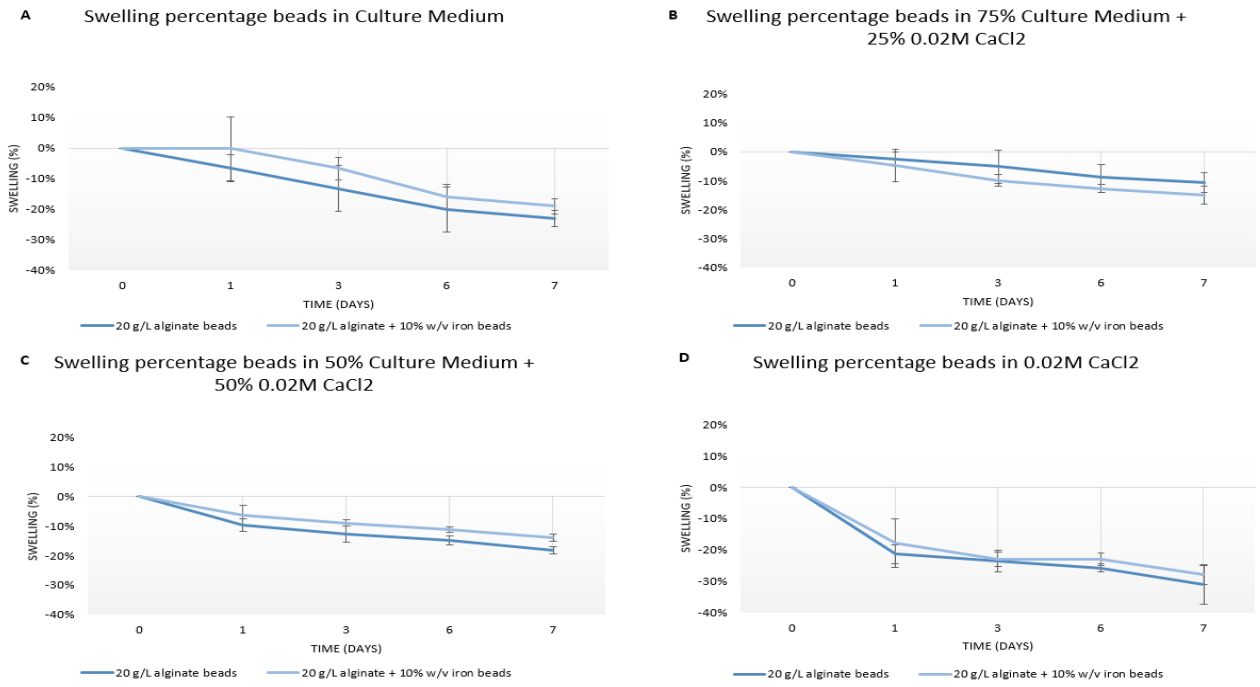


FIGURE 14: SWELLING PERCENTAGES OF ALGINATE BEADS IN A) 100% CM B) 75% CM + 25% 0.02M $CaCl_2$ C) 50% CM + 50% 0.02M $CaCl_2$ D) 100% 0.02M $CaCl_2$

Over the four graphs it is noted that an increase in the fraction of 0.02M $CaCl_2$ results in a decrease of the size of the beads over time. While taking in consideration the standard deviation, no notable differences between the 20 g/L alginate beads and 20 g/L alginate + 10% w/v iron-loaded beads conditions are seen. Figure 15 shows the alteration of size of a 20 g/L alginate bead in 0.02M $CaCl_2$.

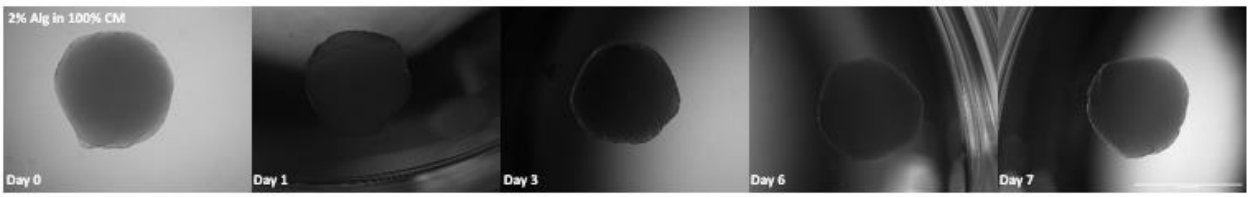


FIGURE 15: 20 G/L ALGINATE BEAD IN 100% 0.02M $CaCl_2$ MEDIUM FROM DAY 0 TO DAY 7. SCALEBAR IS 2000µM

A statistical test was performed over the total swelling percentage to determine if there was a significant difference between the alginate and iron-loaded beads. The executed method is a T-test, that evaluates the statistical significance between two variables, iron-loaded and non-iron-loaded. The p-value represents a value between 0 and 1. When the p-value is <0.05, there is a significant difference between both conditions. Table 5 shows that this is not the case in this study.

Condition	P-value
100% CM	0.106
75% CM + 25% 0.02M $CaCl_2$	0.216
50% CM + 50% 0.02M $CaCl_2$	0.374
100% 0.02M $CaCl_2$	0.519

TABLE 5: P VALUES FROM THE T-TEST FOR THE OVERALL CHANGE IN SWELLING OVER 7 DAYS

The decrease in size due to the fraction of 0.02M $CaCl_2$ does not only hold for the size after 7 days, but also for the initial size at day 0. It was noted that the beads in CM were bigger at T0 than the beads in 0.02M $CaCl_2$. As the beads should be in the same order of sizes, another experiment was executed. Because the 100% CM beads were prepared first, the beads were already in the well for ± 1 hour before the first image. Therefore, it was interesting to examine the behavior of the beads within the first hour in CM. Two beads in CM were analyzed over one hour. An image was taken of both beads in ethanol and every ten minutes from T0 to T60 in CM. The first bead increased 51.06% in size in the first hour in CM and the second bead increased even further, 63.29%, after the first hour in CM. Figure 16 shows the alteration in size of the second bead.

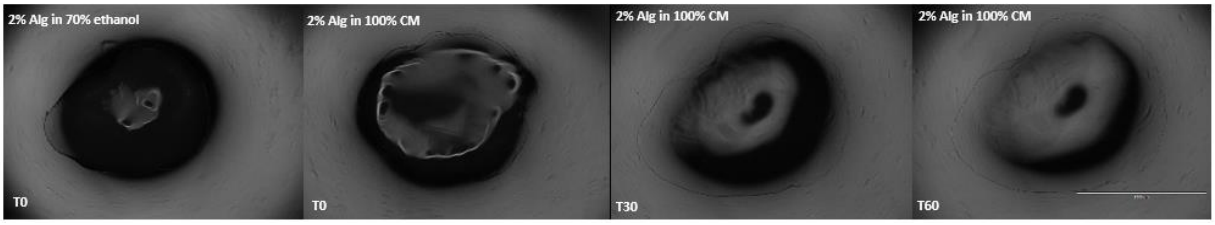


FIGURE 16: SWELLING ANALYSIS OF 20 G/L ALGINATE BEADS IN CM FOR ONE HOUR. THE SCALEBAR IS 2000µM

Both of these swelling studies, the first hour and the 7 days can be combined to illustrate the swelling behavior of a 20 g/L alginate bead in 100% CM. Figure 17 shows how both beads first swell fast and then over time decrease in size. The first 20 minutes in the medium have the most impact on the swelling. After that, the swelling of the beads slowly keeps increasing till minute 60. Note that this is a combination of results of the second experiment in section 2.2.3 and the first hour experiment. In section 3.2, another size analysis was done over 5 days, including the first-hour results as well. This showed similar results to the two samples in Figure 17.

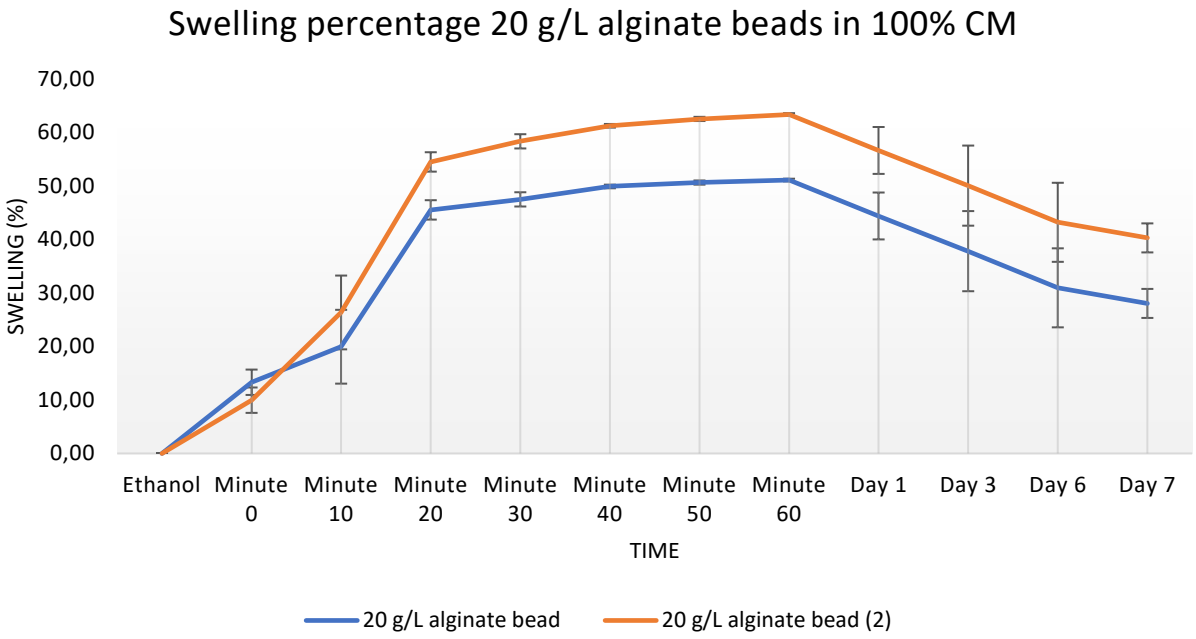


FIGURE 17: TOTAL SWELLING PERCENTAGE OF TWO 20 G/L ALGINATE BEADS IN CULTURE MEDIUM IN THE FIRST 60 MINUTES AND 7 DAYS FOLLOWING

3.2 MECHANICAL PROPERTIES STUDY

The mechanical study of the alginate beads considered eight conditions. Two different beads, 20 g/L alginate and 20 g/L alginate + 10 w/v iron were incubated in four different mediums. The mediums were 70% ethanol in which the beads are kept after production, 100% CM, 100% 0.02M $CaCl_2$ and 50% CM + 50% 0.02M $CaCl_2$. The beads were placed in mediums for 5 days prior to the analysis. The 70% ethanol beads were kept in the 4°C fridge. The other three sorts of beads in the medium were placed in petri dishes with 2.5 mL of medium and incubated in the 37°C incubator. Each condition had four beads, whose sizes were analyzed over time.

The rheometer was used to determine the gap and axial force. This was done according to the protocol described in section 2.4.The minimal gap used for the beads in ethanol and CM was 1000µm, 800µm for the beads in 50% CM + 50% 0.02M $CaCl_2$ and 600µm for the beads in 0.02M $CaCl_2$. Figure 18 shows a 10% w/v iron-loaded alginate bead in 100% CM before the test started and afterward.

FIGURE 18: 20 G/L ALGINATE + 10% W/V IRON BEAD IN 100% CM BEFORE AND AFTER RHEOMETER TEST

The rheometer test measured the gap and axial force every 0.5s, the smallest interval possible on the rheometer. From the gap and axial force of the rheometer test and the known diameter (later computed to radius) from the size analysis, the Young's Modulus could be computed with equations (1) and (2). A note for the results is that the 50% CM + 50% 0.02M $CaCl_2$ condition only had three beads available for analysis.

The size analysis was done approximately 20 minutes before the compression tests. As the beads were not marked and there were four beads per well, it was not possible to tell them apart to know exactly what bead was used for what compression test. Therefore, the average size of the beads is taken to compute the compressive stiffness. Table 6 shows the diameter of the beads and the standard deviation.

Medium	Diameter 20 g/L alginate beads (µm)	Diameter 20 g/L alginate + 10% w/v iron beads (µm)
Ethanol	2397 ± 31	2375 ± 18
100% CM	3073 ± 49	3074 ± 61
100% 0.02M $CaCl_2$	1946 ± 27	1964 ± 31
50% CM + 50% 0.02M $CaCl_2$	2219 ± 239	2332 ± 33

TABLE 6: DIAMETER OF THE BEADS USED FOR THE RHEOMETER TEST

The high standard deviation of the 20 g/L alginate bead in 50% CM + 50% 0.02M $CaCl_2$ was caused by one bead that was 300µm smaller than the other two beads. As mentioned, the size of the beads was also analyzed daily to compare the influence of swelling on the mechanical stiffness as well. Both conditions in the culture medium showed strong swelling in the first minutes and later started to decrease in size after a few days. The alginate condition was at 59.50% swelling after 5 days, whereas the iron-loaded beads was at 60.13% swelling after 5 days. The 0.02M $CaCl_2$ conditions both started with a small increase in size but eventually lost 35.49% and 30.25% in size. The same behavior was observed in the 50% CM + 50% 0.02M $CaCl_2$ conditions, where the beads started to swell in the first minutes, but eventually decreased by 12.12% for the alginate bead and decreased 4.01% for the iron-loaded beads on day 5.

The results of the axial force and displacement are plotted against each other from which the slope was calculated. Both graphs have an R^2 value of >0.99, showing the linearity of the results. Figure 19 shows two examples, one from a bead in 100% CM and one in 100% 0.02M $CaCl_2$.

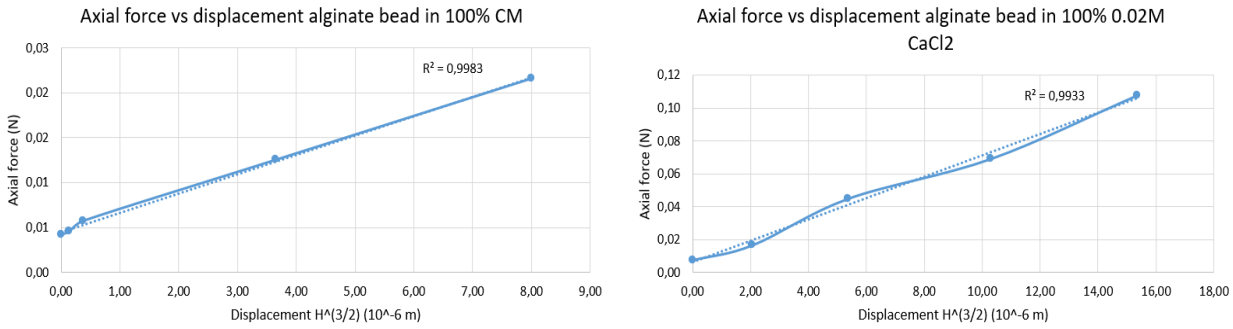


FIGURE 19: AXIAL FORCE VERSUS DISPLACEMENT FOR AN ALGINATE BEAD IN CM AND AN ALGINATE BEAD IN 0.02M $CaCl_2$.

The distribution of the compressive stiffness between the two different beads per medium and their swelling percentage at day 5 is shown in Figure 20.

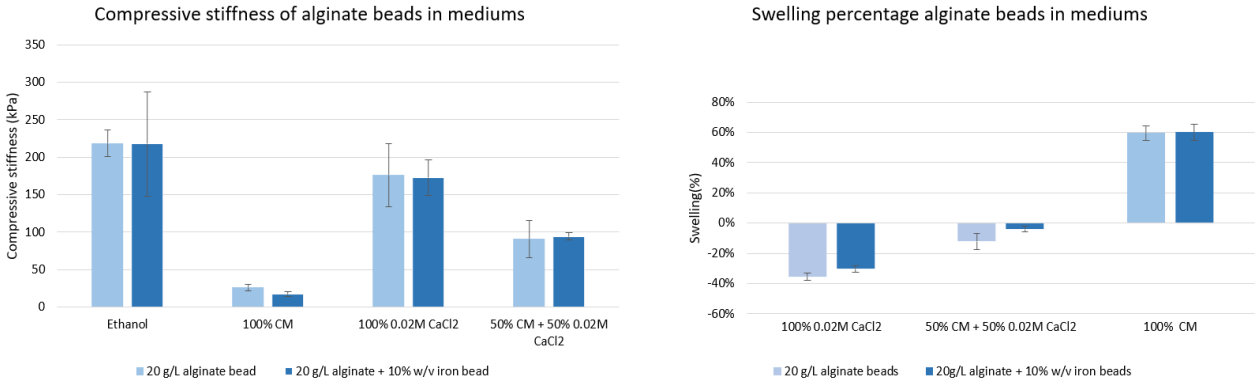


FIGURE 20: COMPRESSIVE STIFFNESS AND SWELLING PERCENTAGE OF BOTH BEADS IN DIFFERENT MEDIUMS AT DAY 5

The results from Figure 20 show that the most swollen 100% CM beads have the lowest compressive stiffness, ranging from 21.88 – 30.59 kPa for the alginate beads and from 14.71 – 20.94 kPa for the iron-loaded alginate beads. The shrunken 0.02M $CaCl_2$ beads have much higher compressive stiffness. The 20 g/L alginate beads have the two highest and lowest compressive stiffness from this medium ranging from 118.95 – 214.40 kPa, while the iron-loaded beads stay in the middle with 144.50 – 192.67 kPa.

The strongest beads come from the 70% ethanol medium. There, the alginate beads are stable between 202.9 – 244.26 kPa and the iron-loaded alginate beads start at 159.51 – 304.44 kPa, making it a wide interval. The most swollen beads have the lowest compressive stiffness, while the most shrunken beads are not the strongest. The combination of CM and $CaCl_2$ makes the compressive stiffness higher than the beads in CM, but lower than the $CaCl_2$ condition. Here, the weakest bead is at 70.11 kPa, going up to 117.68 kPa for the strongest bead.

As in the swelling study, there was a T-test executed for the mechanical strength study. The P-values are given in Table 7. This time, only the 100% CM condition shows a statistically significant result. Meaning that there is a significant difference between the results of the alginate and iron-loaded alginate beads.

Condition	P-value
70% Ethanol	0.985
100% CM	0.020
100% 0.02M $CaCl_2$	0.910
50% CM + 50% 0.02M $CaCl_2$	0.822

TABLE 7: P-VALUES FROM T-TEST OVER THE COMPRESSIVE STIFFNESS AT DAY 5

4 DISCUSSION

4.1 MORPHOLOGY AND SWELLING STUDY

The results have shown that the beads of the first experiment all started with a sphericity >95%. The beads from the second medium experiment had lower sphericity, from 92-96%. The fixed collecting distance of 3.5 cm, the addition of 70% ethanol to the crosslinking solution and shaking of the flask to lower the surface tension did help in the production of spherical and pear-shaped beads[14,16]. The 23G nozzle did make beads that were 2.6-2.8 mm and the 27G nozzle did make beads that were 2.3-2.4 mm as expected.

Changes in sphericity over time can be explained by their swelling behavior, which affects the beads' chemical stability. As the wells in the first experiment contained around 10 beads per well and 4 were analyzed daily, there were some unexplainable fluctuations in the sphericity. Some conditions decreased in sphericity one day and gained sphericity the next day, due to different beads that were analyzed over time. This induced higher standard deviations, resulting in no significant differences between conditions. Due to the fluctuations and high errors, the second medium experiment only contained one bead per well. In this way, the same beads were analyzed every time.

Both combinations of alginate and iron-loaded alginate beads in PBS did show swelling from 8% to 45% in the first experiment and even dissolved completely in both conditions after one day in the second experiment. PBS contains positively charged ions, which will compete with the available Ca^{2+} and connect to the negatively charged carboxylate ion of the alginate structure. This increases the electrostatic repulsion, followed by chain relaxations and so causing the swelling of the alginate beads[19]. The calcium ions also get into competition with present phosphates, which makes it possible for the negative carboxylate group to attach to other positively charged ions now less Ca^{2+} is available, causing chain relaxations. The swelling did not influence the sphericity of the first experiment, where all conditions with PBS remained constant in sphericity.

As the beads did not swell in both experiments in CM and combinations with 0.02M $CaCl_2$ over the 7 days. The analysis was done again over the first hour in the medium after production. This time, the beads in CM did show the expected swelling. When the alginate beads were in 100% culture medium, the same reaction occurred as with PBS. Here, the calcium ions get into competition with phosphates too. [19,20]. A difference between PBS and CM is that the CM also contains Ca^{2+} ions, which can counterbalance the influence of the phosphates[21]. When the CM and 0.02M $CaCl_2$ mediums were combined, it was noted that the swelling percentage decreased over time when the fraction of 0.02M $CaCl_2$ increased. Figure 14 illustrates this decrease in swelling when the fraction of 0.02M $CaCl_2$ is increased. This decrease is explainable by the density of the alginate chains. When more Ca^{2+} is available, the crosslinking density increases[8,22]. The eggbox model (Figure 3) becomes more compact when the crosslinking density increases and the size of the beads will decrease. The sphericity was not impacted in any CM or $CaCl_2$ condition by the changes in size. The maximum difference, with the standard deviation included, was not bigger than 0.31%. Leaving the change in sphericity negligible.

In the second medium experiment, the 50% 0.02M $CaCl_2$ + 50% PBS medium showed precipitation. This was caused by the presence of phosphates in PBS. Phosphates and calcium ions can precipitate when available in high concentrations[23].

4.2 MECHANICAL PROPERTIES STUDY

To obtain usable results, the protocol for the rheometer tests had to be changed from the way references recommended. The gap and pressing speed were the adjustable parameters in this test. According to Wang et al., the compressive stiffness of alginate microspheres increases when the compressing speed is increased[24]. A compressing speed starting from around 500 µm/s would be the optimal way for a compressive test on alginate beads. However, our rheometer has a minimum interval of 0.5 seconds, making it not possible to gain information from the results. When a compressive speed of 500µm/s or faster were tried on the alginate beads (1900µm to 3070µm in size), there were not enough data points available, as the beads also should not be totally compressed. For this reason, the 300µm/s was chosen. In this way, enough data points were given to be able to analyze the data. Next to the compressive speed, the gap was also an important parameter. The optimal way, according to Wang et al., was a maximum deformation of 50%[24]. Again, because of the time interval for data points and a fitting compressive speed, this bound was increased to approximately 66%. 600µm for the 1900µm beads, 800µm for the 2400µm beads and 1000µm for the beads around 3000µm. Normally this bottom line is set at 50% because after 50% deformation, the beads are not able to recover to their initial form[24]. This higher deformation resulted in beads that were compressed to a non-recoverable state but did present an insight into the mechanical strength of the beads.

The results showed that the swollen beads in 100% culture medium have the lowest compressive stiffness. Starting from 14.71 kPa to 20.94 kPa for the iron-loaded alginate beads and the alginate beads ranged from 21.88 kPa to 30.59 kPa. The CM beads of both conditions showed no elastic behavior over all tests. Because of their high swelling percentage, the beads became very fragile, which is also represented by the low compressive stiffness[21,25]. This meant that after compression, the structure was lost and the beads busted. This medium showed a significant difference between both conditions. Where the swelling percentage was almost the same for both, the alginate beads had higher compressive stiffness.

The beads in 0.02M $CaCl_2$ showed much higher compressive stiffness than the beads in CM. As mentioned in section 4.1, the influence of Ca^{2+} on the stability and swelling behavior has an impact on the compressive stiffness as well. The increased crosslinking density makes the alginate structure stiffer and stronger, leading to a higher Young's Modulus. Ranging from 118.95 to 200.63 kPa for the alginate beads and making the average almost 7 times higher than the alginate beads in CM. The iron-loaded alginate beads are on average 10 times higher than in CM, ranging from 144.5 to 192.67 kPa. The beads in $CaCl_2$ showed no elastic behavior after compression but broke down, meaning that the structure was not totally lost.

As expected, the combination of 50% CM and 50% 0.02M $CaCl_2$ showed results sitting between the 100% CM and 100% 0.02M $CaCl_2$ conditions. This is also clearly shown in Figure 20. This result is confirmed by the swelling behavior, where after 5 days the beads had decreased in size by 12% for the alginate beads and 4% for the iron-loaded alginate beads. Making them denser in crosslinking and thus stronger than the beads in CM and less dense than the beads in 0.02M $CaCl_2$[8,22]. The average compressive stiffness was 90.64 kPa for the alginate beads and 94.27 kPa for the iron-loaded alginate beads with a p-value of 0.822, meaning no significant difference was present between the results.

5 CONCLUSION

Based on the results of this study, it can be concluded that the surrounding medium does impact the chemical stability of the alginate and iron-loaded alginate beads. By following the morphological and mechanical properties of the beads over the course of 7 days and in different medium compositions, the beads showed similar behavior towards the mediums throughout the study.

Over time, it can be said that the swelling has the most influence on the chemical stability of the alginate and iron-loaded alginate beads. Swelling is mostly caused by the exchange of ions, which in turn also causes a decrease in stability. The ion exchanges weaken the structured alginate compound crosslinked with $CaCl_2$ by causing chain relaxations and the possibility for other ions to link with the negative carboxylate groups of alginate. The morphology study concluded that the choice of medium and the following swelling behavior did not impact the sphericity over time. At the moment the swelling percentage becomes too high or the alginate structure gets too weak, the bead dissolves instead of losing shape.

It is concluded that the mechanical tests confirm this behavior. Where the highly swollen cell culture medium beads are very fragile and even destructible at the slightest contact, the alginate beads incubated in lower calcium chloride concentration result in a decreased swelling percentage and are seven to ten times stiffer and stronger.

The comparison between the 20 g/L alginate beads and 20 g/L alginate + 10% w/v iron-loaded beads concerning their response to the different mediums provided a lack of significant difference in swelling percentages across the different mediums. Also, the executed t-test demonstrated that the small variations in swelling percentages and sphericity are not statistically significant.

In conclusion, the impact of the surrounding medium on the chemical stability is high. The stability can be greatly reduced or increased by the choice of medium. To make the iron-loaded alginate beads suitable for local mechanical stimulation, the fractions of cell culture medium, $CaCl_2$ and eventual extra additions should be perfectly balanced before there is a chance for an in vivo use with suitable properties.

6 REFERENCES

[1] Biomaterials. National Institute of Biomedical Imaging and Bioengineering n.d. https://www.nibib.nih.gov/science-education/science-topics/biomaterials (accessed December 23, 2023).

[2] Pavlovic M. What Are Biomaterials? Bioengineering: A Conceptual Approach. 2015:229–44. doi:10.1007/978-3-319-10798-1_18.

[3] Park JB. Biomaterials Science and Engineering. Springer Science & Business Media. 2012.

[4] Olson JL, Atala A, Yoo JJ. Tissue Engineering: Current Strategies and Future Directions. Chonnam Med J 2011;47:1–13. doi:10.4068/cmj.2011.47.1.1.

[5] Salinas EY, Hu JC, Athanasiou K. A Guide for Using Mechanical Stimulation to Enhance Tissue-Engineered Articular Cartilage Properties. Tissue Eng Part B Rev 2018;24:345–58. doi:10.1089/ten.teb.2018.0006.

[6] Zhang Y, Habibovic P. Delivering Mechanical Stimulation to Cells: State of the Art in Materials and Devices Design. Advanced Materials 2022;34:1-18,2110267. doi:10.1002/adma.202110267.

[7] Iskratsch T, Wolfenson H, Sheetz MP. Appreciating force and shape — the rise of mechanotransduction in cell biology. Nat Rev Mol Cell Biol 2014;15:825–33. doi:10.1038/nrm3903.

[8] Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci 2012;37:106–26. doi:10.1016/j.progpolymsci.2011.06.003.

[9] Martínez Cano B, Mendoza-Meneses C, García-Trejo J, Macías-Bobadilla G, Aguirre-Becerra H, Soto-Zarazúa G, et al. Review and Perspectives of the Use of Alginate as a Polymer Matrix for Microorganisms Applied in Agro-Industry. Molecules 2022;27:4248. doi:10.3390/molecules27134248.

[10] Orive G, Ponce S, Hernández RM, Gascón AR, Igartua M, Pedraz JL. Biocompatibility of microcapsules for cell immobilization elaborated with different types of alginates. Biomaterials 2002;23:3825–31. doi:10.1016/S0142-9612(02)00118-7.

[11] Farshidfar N, Iravani S, Varma RS. Alginate-Based Biomaterials in Tissue Engineering and Regenerative Medicine. Mar Drugs. 2023;21:189. doi:10.3390/md21030189.

[12] Akila M, Souhila B, Drouiche N, Adour L, Lounici H. Cross-linking and modification of sodium alginate biopolymer for dye removal in aqueous solution. Polymer Bulletin. 2019:76. doi:10.1007/s00289-018-2557-x.

[13] Rivera A. Master Thesis Development of magnetic alginate beads for localized mechanical stimulation in tissue engineering. University of Twente. 2023. https://essay.utwente.nl/97705/1/Rivera_MA_TNW.pdf

[14] Lee B-B, Ravindra P, Chan E-S. Size and Shape of Calcium Alginate Beads Produced by Extrusion Dripping. Chemical Engineering & Technology 2013;36:1627–42. doi:10.1002/ceat.201300230.

[15] Calculator for making ethanol in water solution. n.d. https://tinyurl.com/handymath (accessed November 16, 2023).

[16] Patra M, Salonen E, Terama E, Vattulainen I, Faller R, Lee BW, et al. Under the Influence of Alcohol: The Effect of Ethanol and Methanol on Lipid Bilayers. Biophys J 2006;90:1121–35. doi:10.1529/biophysj.105.062364.

[17] Chan E-S, Lim T-K, Voo W-P, Pogaku R, Tey BT, Zhang Z. Effect of formulation of alginate beads on their mechanical behavior and stiffness. Particuology 2011;9:228–34. doi:10.1016/j.partic.2010.12.002.

[18] Davarcı F, Turan D, Ozcelik B, Poncelet D. The influence of solution viscosities and surface tension on calcium-alginate microbead formation using dripping technique. Food Hydrocolloids 2017;62:119–27. doi:10.1016/j.foodhyd.2016.06.029.

[19] Bajpai SK, Sharma S. Investigation of swelling/degradation behaviour of alginate beads crosslinked with Ca2+ and Ba2+ ions. Reactive and Functional Polymers 2004;59:129–40. doi:10.1016/j.reactfunctpolym.2004.01.002.

[20] Maiti S, Singha K, Ray S, Dey P, Sa B. Adipic acid dihydrazide treated partially oxidized alginate beads for sustained oral delivery of flurbiprofen. Pharm Dev Technol 2009;14:461–70. doi:10.1080/10837450802712658.

[21] Chui C-Y, Bonilla-Brunner A, Seifert J, Contera S, Ye H. Atomic force microscopy-indentation demonstrates that alginate beads are mechanically stable under cell culture conditions. Journal of the Mechanical Behavior of Biomedical Materials 2019;93:61–9. doi:10.1016/j.jmbbm.2019.01.019.

[22] Tavakol M, Vasheghani-Farahani E, Hashemi-Najafabadi S. The effect of polymer and CaCl2 concentrations on the sulfasalazine release from alginate-N,O-carboxymethyl chitosan beads. Prog Biomater 2013;2:10. doi:10.1186/2194-0517-2-10.

[23] Larson MC, Luthi MR, Hogg N, Hillery CA. Calcium-phosphate microprecipitates mimic microparticles when examined with flow cytometry. Cytometry A 2013;83:242–50. doi:10.1002/cyto.a.22222.

[24] Wang CX, Cowen C, Zhang Z, Thomas CR. High-speed compression of single alginate microspheres. Chemical Engineering Science 2005;60:6649–57. doi:10.1016/j.ces.2005.05.052.

[25] Kaklamani G, Cheneler D, Grover LM, Adams MJ, Bowen J. Mechanical properties of alginate hydrogels manufactured using external gelation. Journal of the Mechanical Behavior of Biomedical Materials 2014;36:135–42. doi:10.1016/j.jmbbm.2014.04.013.