University of Zaragoza

Faculty of Sciences

Biotechnology Bachelor´s Degree

Hydrocolloids of egg white and gelatin as a platform for hydrogel-based tissue engineering: a feasibility study

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INDEX

Abstract

One important aspect of tissue engineering is finding a biomaterial able to act as a scaffold and provide a structural framework for cell attachment and growth. Among the polymeric materials used as scaffolds, natural polymers present many advantages compared to other materials, as they can mimic extracellular matrix elements and provide a microenvironment suitable for cell growth and proliferation. Chicken egg white has shown to be a native biomaterial with interesting structural, biological and physico-chemical properties

This work tests the feasibility of using hydrocolloids of egg white and gelatin as a platform for hydrogel-based tissue engineering. Hydrocolloids were prepared, and their rheological properties were studied. These solutions were used for the study of cell culture and viability in 2D surfaces and 3D miniaturised environments created using microfluidic devices. They were also used for the fabrication of 3D hydrogel scaffolds, and the study of its micro- and macro- structural properties.

Results showed that these hydrocolloids of egg white ang gelatin could be successfully used for hydrogel-based tissue engineering. This work serves as a first step for future research of its application in the biomedical field. This would allow the development of biological substitutes for the treatment of organ and tissue functionality in humans, and also building 3D models to study cellular phenomena.

1. Introduction

1.1. Tissue engineering

Tissue engineering is an interdisciplinary research field that applies the principles of biology and engineering to the development of biological substitutes in order to restore, keep or improve organ and tissue functionality in the human body (1).

For the development of the tissue construct, three principal elements are needed: a 3D biomaterial that works as a scaffold for the cells to hold on to for their proliferation and molecular/environmental signals used by the cells to grow and perform other cellular functions [Figure 1].

Tissue engineering has many applications, one of the most common being the study of organ and tissue development as well as their possible malformations, which contributes to the development of new therapies. It also has other applications, such as *in vitro* modelling of diseases, in order to improve personalized medicine, and even climate change contributions with the development of meat and fish made in the laboratory (2).

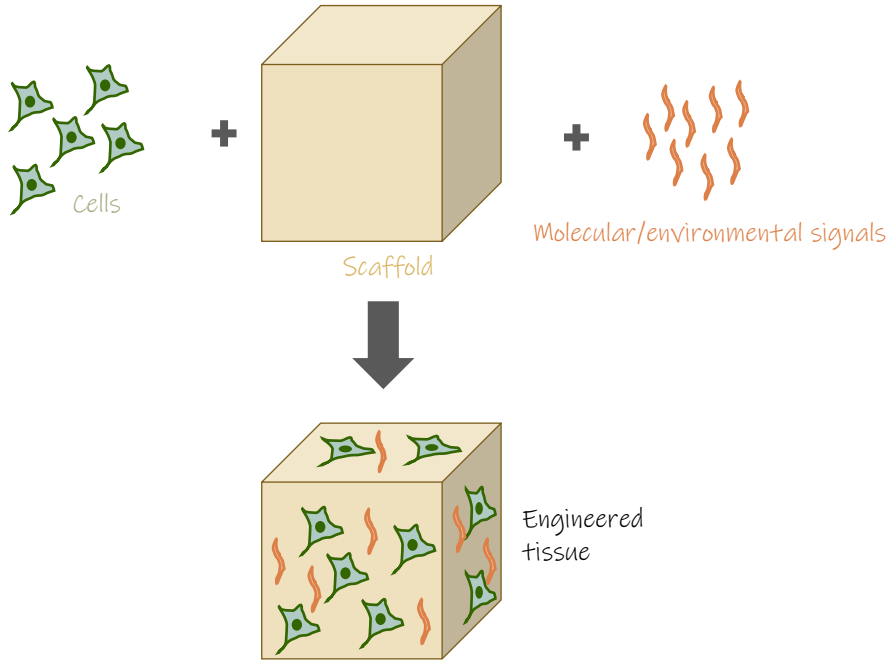


Figure 1: Tissue engineering paradigm consists of the isolation and proliferation of cells by seeding them on a scaffold made from a 3D biomaterial and stimulating them with molecular/environmental signals.

1.2. Biomaterials for tissue engineering

Biomaterials are an important component of tissue engineering, as they are designed to act as scaffolds and provide a structural framework for cell attachment and growth (3). There are many biomaterials that can be used in this field [Table 1], metallic and ceramic biomaterials being widely used in biomedicine. Polymeric materials, natural and synthetic, have properties that make them attractive for their biomedical applications, such as the versatility of their chemistry, physico-mechanical properties, manufacturability and degradation rates (4). In recent years, carbon is attracting great attention as a biomaterial for tissue engineering too.

Even though all these biomaterials have their advantages, when improved mechanical, physico-chemical and biological properties are required, a combination of them can be used, namely composites, in order to minimize every possible drawback (5)(6).

Table 1: Biomaterial composition of tissue engineering scaffolds (4).

Biomaterial		Examples
Polymeric material	Natural molecules	Proteins (collagen, fibrin) Polysaccharides (alginate, hyaluronic acid) Polynucleotides (DNA, RNA)
	Synthetic molecules	Polyesters (polycaprolactone, poly lactic acid), polyurethane
Ceramics		Calcium phosphate, bioactive glasses
Metals		Titanium or magnesium and their alloys
Carbon		Graphene, nanotubes or nanofibers

Among polymeric materials, natural polymers have been preferred over synthetic polymers in various biomedical studies, due to their several advantages. They mimic extracellular matrix (ECM) elements, and they present peptide sequences, protease cleavage or integrin cell-binding sites, all of which results in improved cellular behaviour *in vitro* and *in vivo* (7)(8)(9). Polymers of greatest interest are those with high availability and bioactivity, easy to handle and with a lower production cost. For instance, protein-based biomaterials are bioactive and support cellular responses (attachment, migration, proliferation), but they have limited sources and their extraction and purification procedures are complex, making them costly (10). However, egg white is an overlooked protein-based material that can be used in different applications, thanks to its easy-availability and low cost.

1.3. Egg white

Chicken egg white (EW) is a native biomaterial with interesting structural, biological and physico-chemical properties. It has an intrinsic liquid phase that makes it needless of any additional resuspension into a solution. Its viscosity can be modulated by temperature and its transparency makes it suitable for 3D culture systems (11)(12). Being rich in components with high biological activities, EW is suitable for various biomedical applications, due to its antibacterial, antihypertensive, antiinflammatory, healing-enhancing and cell growth stimulatory features (13).

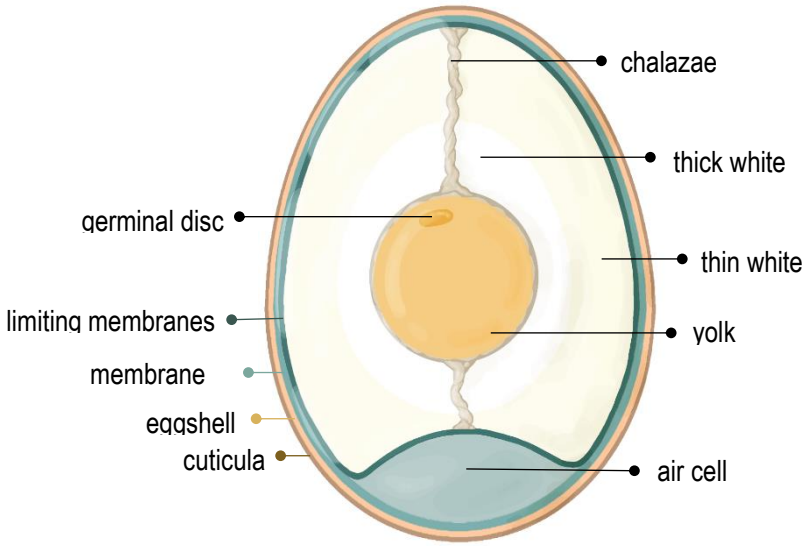


Figure 2: The major structural components of chicken egg are shell, shell membrane, yolk and white. Eggshell is composed of three layers; egg white being found inside of it and eggshell membrane between the two of them. Yolk is suspended in the egg white via the chalazae.

The major structural components of chicken egg are shell, shell membrane, yolk and white. EW is located between eggshell membrane layers and yolk. Eggshell components are calcium and phosphate, and its structure is porous to allow air permeation to the interior. Eggshell membrane, found between the eggshell and EW has a protein-based structure and supports the formation of proteins and enzymes. It has three morphologically distinct layers that protect the egg content from bacteria: outer, inner and limiting membranes (14)(15)(16). Egg yolk is a source of nutrients and vitamins for the embryo, as well as a reservoir of immunoglobulin. It is found suspended in the EW via the chalazae, two connection tissues (17)(18). EW acts as a second protection layer to prevent bacterial infections from reaching the yolk [Figure 2].

EW is a mixture of water (~85%), proteins (~10%) and carbohydrates and is composed of four layers that differ in their viscosities, named based on their viscosity and position in respect to yolk. Thus, from the outside in, the following are found: outer thin, outer thick, inner thin and inner thick. High content of ovomucin is responsible for EW's high viscosity, which decreases with increasing temperature. The emulsion stability, as well as proteins' emulsifying activity depend on pH, salt presence and protein concentration (19).

Proteins are the principal element of EW, contributing to its physical and biological properties, which makes them ideal bioactive compounds for medical, pharmaceutical and bioengineering applications [Figure 3] (20). They are globular and categorized into two groups according to their abundance: main proteins (>83%) and minor proteins (<17%).

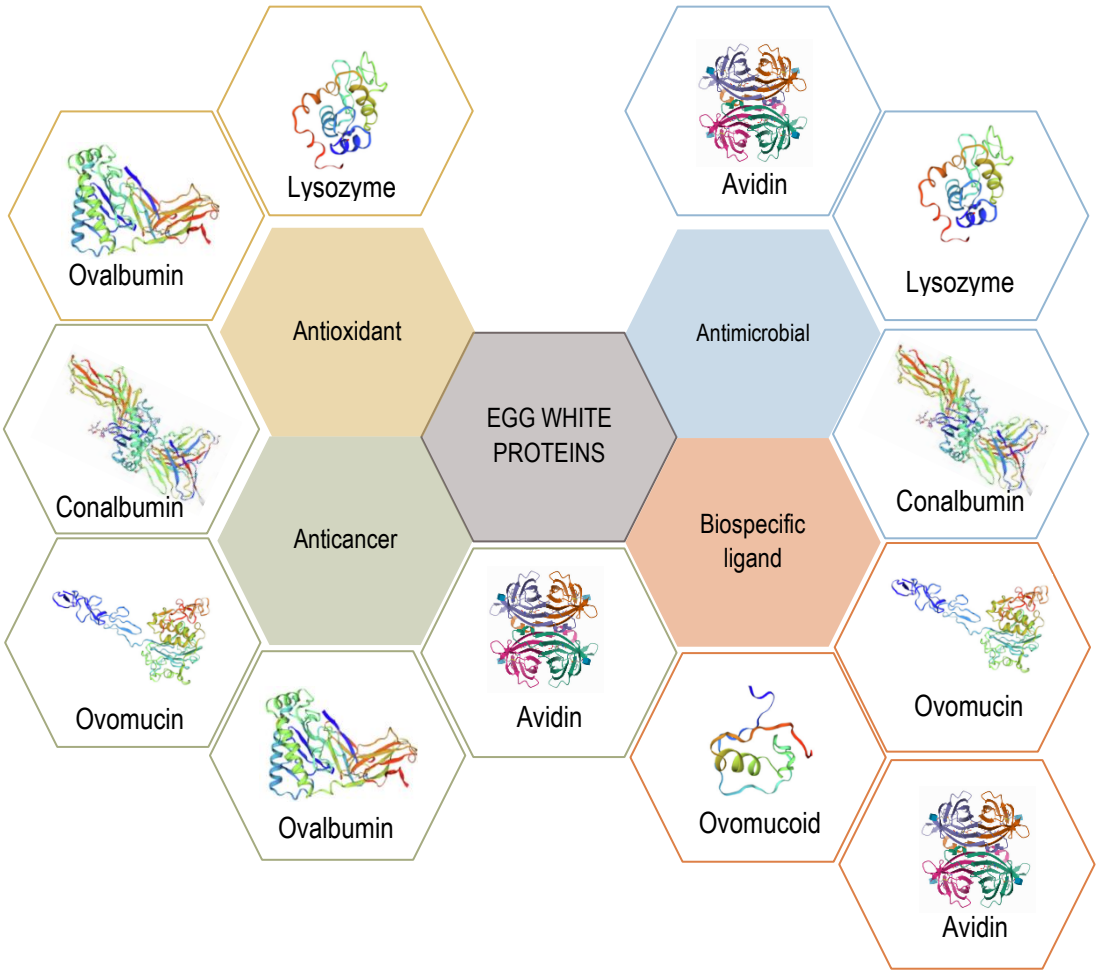


Figure 3: EW proteins contribute to its physical and biological properties some of which include antimicrobial, antioxidant, anticancer and biospecific ligand.

1.3.1. Main proteins

Ovalbumin (OVA)

Ovalbumin comprises 54% of total EW proteins (21). OVA is a phosphoglycoprotein with an important role in heat-induced gelation, foaming and emulsifying properties of EW. However, its structure and properties can change over storage time, a proportion of it being irreversibly converted into S-ovalbumin, which is more heat-stable (22).

Conalbumin/Ovotranferrin

Conalbumin comprises 13% of total EW proteins (21). It is a glycoprotein with 15 disulphide bonds that provide structural stability, and iron biding capability providing antimicrobial properties. Conalbumin is the most heat-sensitive protein, its denaturation and aggregation leading to alteration of EW viscosity and initial gelation. Yet, this aggregation can be inhibited by OVA (22).

Ovomucoid (OM)

Ovomucoid comprises 11% of total EW proteins (21). OM is a glycoprotein that functions as a trypsin inhibitor. Furthermore, due to its resistance to heat and enzyme digestion and its allergic reactivity, OM is a featured allergen in EW (22).

Lysozyme

Lysozyme comprises 3.4% of total EW proteins (21)c. It´s a secretory enzyme with 4 disulphide bonds which confer stability as well as cohesion. This enzyme has antimicrobial properties because it catalyses the hydrolysis of bacterial cell walls´ peptidoglycan. Furthermore, it is positively charged, unlike other EW properties, so it is able to interact with negatively charged molecules (22).

1.3.2. Minor proteins

Regardless of their low abundance, there are other proteins, found in different concentrations, that are influential for EW´s physicochemical and biological characteristics (23). Some of the most relevant ones are ovoinhibitor (1.5%), ovoglycoprotein (1%), flavoprotein (0.8%), ovomacroglobulin (0.5%), cystatin (0.05%) and avidin (0.05%) (24).

1.4. Gelatin

Gelatin is a natural animal protein obtained by acid or alkali catalysed hydrolysis of collagen connective tissue of muscles, skin and bones of animals. It has many attractive properties, like biodegradability, biocompatibility, non-antigenicity, plasticity and adhesiveness (25). Gelatin hydrogels are stabilized by hydrogen and electrostatic bonds, as well as hydrophobic interactions. They are thermally reversible, melting around human body temperature, giving it melt-in-mouth properties. This gelling agent is used for different biological and functional purposes in pharmaceutical, biomedical, cosmetic and food formulations(26).

1.5. Gelation

Gelation is the formation of a continuous 3D network by chemical or physical crosslinking that traps and immobilizes the liquid within it to yield a polymeric structure of infinite viscosity, i.e. a gel that resists flow under pressure. When the trapped liquid is water, a hydrogel is yielded. Hydrogels hold great potential for tissue engineering and regenerative medicine as they mimic the natural extracellular matrix (ECM), due to their hydrated nature, viscoelastic properties and ability to incorporate cells or growth factors.

EW is a famous heat-induced gel, which is denatured by heating to form an ordered gel network structure that is formed by hydrophobic interaction and is irreversible. This ability of EW to gel is an important functional property which plays an important role in the food industry and its derived products (e.g. dairy products, jelly, sausage and gel products). Gelatin has thermoreversible properties, i.e. when the gelatin solution is cooled below the sol-gel transition temperature, triple helix from spiral molecules are formed

structuring into an elastic hydrogel, while in hot water hydrogen and electrostatic bonds are decomposed, soluble collagen is denatured, and the molecules produce a hydrocolloid. It has been shown that when gelatin is mixed with other substances at certain concentrations, the resulting mixtures also show thermoreversible properties.

As just described, the gel properties of EW as well as those of gelatin have been studied and reported. However, the gel properties of mixed EW and gelatin hydrocolloids have not received nearly the same attention. Indeed, mixtures of EW and gelatin have been investigated for food industrial applications but to the best of our knowledge, have not been reported for tissue engineering applications (11)(12)(13).

2. Objectives

The general objective of this work consists in testing the feasibility of using hydrocolloids of egg white and gelatin for hydrogel-based tissue-engineering. This work is based on different experiments to determine the properties of the mixed hydrocolloids, as well as the cell viability and proliferation of cultures established on them. These cultures were established on 2D surfaces as well as in 3D scaffolds embedded in microfluidic devices. Finally, 3D hydrogel scaffolds were fabricated with the hydrocolloids. This work serves as a first step for biomaterial-based tissue engineering and their potential biomedical applications using hydrocolloids of egg white and gelatin.

2.1. Specific objectives and work plan

- Study of the rheological properties of hydrocolloids of egg white and gelatin.
- Assessment of cell viability and growth on 2D surfaces coated with hydrocolloids of egg white and gelatin.
- Introduction of hydrocolloids of egg white and gelatin in microfluidic devices to create a 3D miniaturised environment.
- Assessment of cell viability and growth in a 3D miniaturised environment of hydrocolloids of egg white ang gelatin.
- Fabrication of 3D hydrogel scaffolds of egg white and gelatin.
- Observation of the macro- and micro-structure of 3D hydrogel scaffolds of egg white and gelatin.

3. Materials and methods

3.1. Egg white lyophilization

Large chicken eggs (*Gallus gallus domesticus*) from a local supermarket were cracked open and the contents placed on a Petri dish. Avoiding the yolk and chalazae, the EW was transferred to a Falcon tube using plastic Pasteur pipettes. About 30ml of EW were collected per egg. EW was lyophilized and a powder of the same volume as the solution was obtained.

3.2. Egg white colloid solutions

Using EW lyophilized powder, the following colloid solutions were prepared in distilled water (dH_2O):

- 1% w/v: 100 mg of egg white in 10ml of dH_2O.
- 5% w/v: 500 mg of egg white in 10ml of dH_2O.
- 10% w/v: 1g of egg white in 10ml of dH_2O.

The solutions were prepared in 15ml Falcon tubes. pH was measured and adjusted to neutral (pH=7.0±0.5) using sodium hydroxide (NaOH) and/or hydrochloric acid (HCl). Once the pH was adjusted, the solutions were used for the coating of well plates, introduction of a hydrogel in microfluidic devices, or fabrication of 3D hydrogel scaffolds.

3.3. Egg white/gelatin colloid solutions

EW and gelatin (Type B, from bovine skin, Merck) solutions were prepared in distilled water (dH_2O). Also, one gelatin only solution was made, to be used as control.

- 1% egg white + 1% gelatin: 100 mg of EW and 100 mg of gelatin in 10 ml of dH_2O.
- 5% egg white + 1% gelatin: 500 mg of EW and 100 mg of gelatin in 10 ml of dH_2O.
- 10% egg white + 1% gelatin: 1 g of EW and 100 mg of gelatin in 10 ml of dH_2O.
- 1% gelatin: 100 mg of gelatin in 10ml of dH_2O.

pH was measured and adjusted to neutral (pH=7.0±0.5) using NaOH and/or HCl. Once the pH was adjusted, the solutions were used for the coating of well plates, introduction of a hydrogel in microfluidic devices, or fabrication of 3D hydrogel scaffolds.

3.4. Rheology

Rheology was carried out at the Department of Science and Technology of Materials and Fluids of the School of Engineering and Architecture of the University of Zaragoza. The colloidal solutions were characterized by rheological assays using a stress-controlled rotational rheometer HAAKE Rheostress 1 (Thermo Fisher Scientific, Waltham, MA, USA). All samples were tested using a cone-plate configuration with a 35 mm diameter and a cone angle of 1º.

The protocol used in the measurements was the following: 500 µl of each sample were pipetted on the lower plate of the rheometer at 5 ºC. Then, the upper plate descended until the gap between both plates was the required by the sensor specifications (0.051 mm). A solvent trap was used to avoid sample dehydration. After allowing the sample to stabilize for 5 minutes at 5ºC, the oscillatory shear test is executed applying a fixed torque of 5 Nm at the frequency of 5 Hz. The storage G', loss modulus G", complex viscosity of each sample are recorded while the temperature gradually increased from 5 to 70ºC at constant heating rate

3.5. Coating of cell culture plates

Wells of 24 and 12 cell culture well plates were coated with the different colloid solutions as follows: enough solution to cover the bottom of the well was added and the plates left at 4°C until used. The excess solution was then removed and the plates washed with 70% ethanol followed by three washes with PBS before cell seeding.

3.6. Microfluidic devices

Microfluidic devices used in this study have previously been described (27)(28). Briefly, soft lithography was used to develop positive SU8 240-µm relief patterns with the desired geometry on a silicon wafer (Stanford University). Polydimethylsiloxane (PDMS, Sylargd 184, Dow Corning GmbH) was mixed at a 10:1 weight ratio of base to curing agent, the solution poured into the SU8 master and degassed to remove air bubbles. The replica-molded layer was trimmed, perforated and autoclaved. Finally, the PDMS device and 35-mm glass-bottom petri dishes (Ibidi) were plasma-bonded.

The device geometry contained a central chamber [Figure 4] into which the hydrogels were deposited by pipetting them through the small posts and channels. Then they were gellified by applying heat (80 °C for 2 hours) followed by cooling at 4 °C overnight. Two side media channels connected to the central chamber ensured hydration and transport of nutrients [Figure 4].

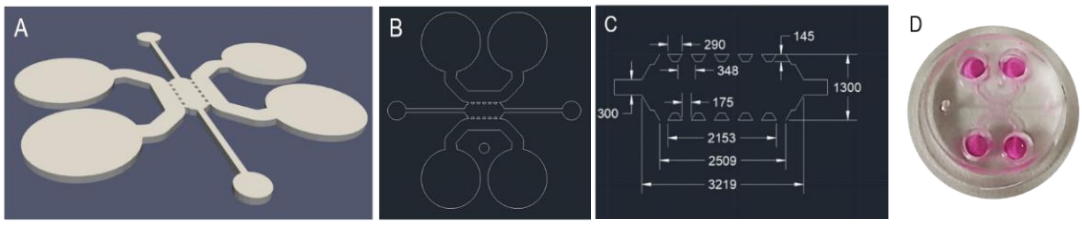


Figure 4: (A and B) Different schemes of the microfluidic device used in this study, which presents a central chamber, 2 small ports and channels for loading of the hydrocolloids and cells, and 4 big ports and 2 big channels for hydration. (C) Dimensions in μm of the central chamber elements. (D) Image of an actual device used in this study (18 mm in diameter).

3.7. Primary cell culture

Primary normal human dermal fibroblasts (pnHDF) were commercially acquired. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Medium was changed every 3 days. Cells were used between passages 4 and 10 while they were proliferative and showed no signs of quiescence. PnHDFs cultures were regularly observed under a phase-contrast light microscope and photographed.

3.8. Cell seeding

On 2D surfaces, 1,000 cells/well for cell viability and proliferation and 10,000 cells/well for cell morphology observation were seeded. About 500 cells/microfluidic device were seeded for both cell viability and proliferation and cell morphology observation.

3.9. Cell proliferation by alamarBlue® assay

Cell proliferation was assessed by the metabolic redox assay alamarBlue® (29). A 24 well plate was used and 10^3 cells were seeded per well. Medium was removed from each well and cells were washed with PBS before adding 1ml of alamarBlue working solution (1/10 alamarBlue®, made up in phenol-free supplemented DMEM) and incubated for 2 hours at 37°C, 5% CO_2. The 1ml samples were transferred to a 96 well plate, by adding 100μl of sample in each well. Triplicate measures of the samples were made. Fluorescence intensity was read (excitation at 530 nm and emission at 590 nm) using a plate reader (Synergy LX, BioTek with Gen5 3.10 software). An N=3 per surface was used.

For microfluidic devices (N=6 per hydrocolloid type), 200μl of alamarBlue working solution were added per chip, which were then incubated for 4 hours at 37°C, 5% CO_2. The 200μl samples were transferred to a 96 well plate, by adding 100μl of sample in each well. Duplicate measures of each sample were made. Fluorescence intensity was read as explained above.

3.10. Phase-contrast light microscopy

Morphology of cell in the different cultures, i.e. 2D coated surfaces (N=3 per surface) and microfluidic devices (N=6 per hydrocolloid type) was observed by phase-contrast light microscopy (DM IL LED, Leica) and photographed.

3.11. 3D hydrogel scaffolds

3D hydrogel scaffolds were fabricated to ~14 mm diameter and ~4 mm thickness. Hydrocolloids used were 1% EW + 1% gelatin and 5% EW + 1% gelatin. Once poured onto the mould, the hydrocolloids were heated at 80°C for 2 hours followed by cooling at 4°C overnight. The resulting gels were chemically cross-linked with either 2.5% or 5% glutaraldehyde (1.04239.0250 Merck) for 4.5 hours at room temperature, followed by 48 hours at 4°C. Scaffolds were thoroughly washed with dH_2O. Finally, the scaffolds were lyophilised.

3.12. Scanning Electron Microscopy (SEM)

An Inspect TM SEM F50 (FEI Company, Hillsboro, OR, USA) in an energy range between 0–30 keV was used to acquire SEM images of 3D scaffolds. Lyophilized samples were coated with a carbon film before examination.

3.13. Data and statistical analysis

Data was analysed with Excel Microsoft software (2016 version). For the alamarBlue results, comparisons between groups were made using one-way analysis of variance with a Holm–Sidak post-hoc analysis (SigmaStat 3.5, San Jose, California). A $p < 0.05$ was considered a significant result.

4. Results

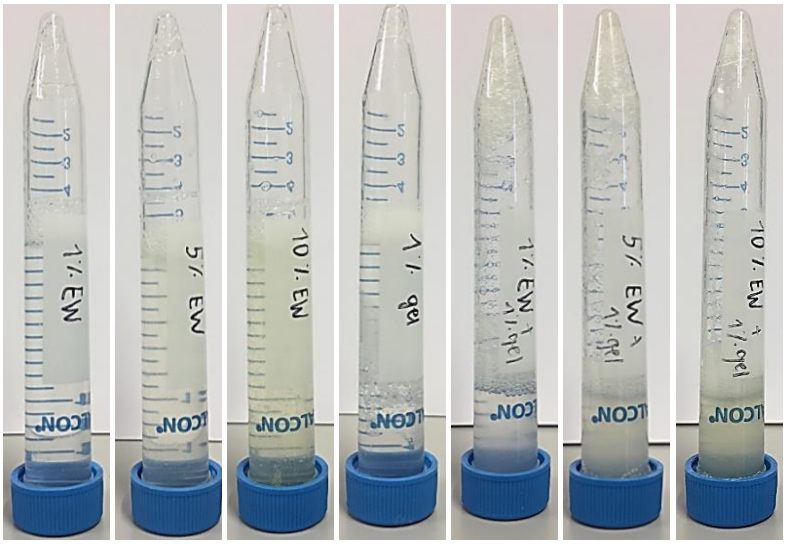
4.1. Lyophilized egg white powder

Lyophilized EW powder appeared yellowish in colour as it can be observed in Figure 5.

Figure 5: Lyophilized egg white powder.

4.2. Colloidal solutions

In terms of the colloidal solutions [Figure 6] the following observations described in Table 2 were made:



A B C D E F G

Figure 6: Colloid solutions with different egg white and gelatin proportions. (A) 1% EW w/v; (B) 5% EW w/v; (C) 10% EW w/v; (D) 1% gelatin; (E) 1% EW w/v + 1% gelatin; (F) 5% EW w/v + 1% gelatin; (G) 10% EW + 1% gelatin.

Table 2: Colloidal solutions observations

Colloidal solution	Observations
1% EW	Liquid, translucid solution.
5% EW	Viscosity in between 1% and 10% w/v solutions, translucid.
10% EW	Thick viscous solution, yellowish in colour and translucid.
1% EW + 1% gelatin	No observable differences compared to 1% EW.
5% EW + 1% gelatin	No observable differences compared to 5% EW.
10% EW + 1% gelatin	No observable differences compared to 10% EW.
1% gelatin	Liquid, translucid yellowish in colour solution.

4.3. pH measurements

It was observed that the pH of EW solutions was alkaline, whilst the introduction of gelatin into the solution decreased the pH (Table 3).

Table 3: initial and adjusted pH of each colloidal solution. Every value is calculated as the average of two experiments.

Solution	pH initial	pH end
1% EW	10.25	7.09
5% EW	10.35	7.42
10% EW	10.38	7.44
1% EW + 1% gelatin	6.31	7.08
5% EW + 1% gelatin	7.33	7.05
10% EW + 1% gelatin	7.62	7.36
1% gelatin	6.71	7.04

4.4. Rheology

Rheology results [Figure 7] showed that G' was higher than G'' for all the solutions, indicating an elastic behaviour over a viscous one.

For the 1% gelatin solution, it was observed that G' and G'' decreased as the temperature increased, indicating that the solution transitioned from a gel to a liquid as the temperature increased. For the EW solutions, the behaviour was the opposite: there was a sol-gel transition as the temperature was increased. For the mixed hydrocolloids, an interesting behaviour was observed: the G' was almost constant until a slight decrease was observed, followed by an increment in G'.

This would indicate that these solutions are mostly a gel until a destabilization of the system is followed by a rearrangement into a more viscous gel. Nevertheless, the recorded signal for G'' was generally very noisy, which would need tuning in future work.

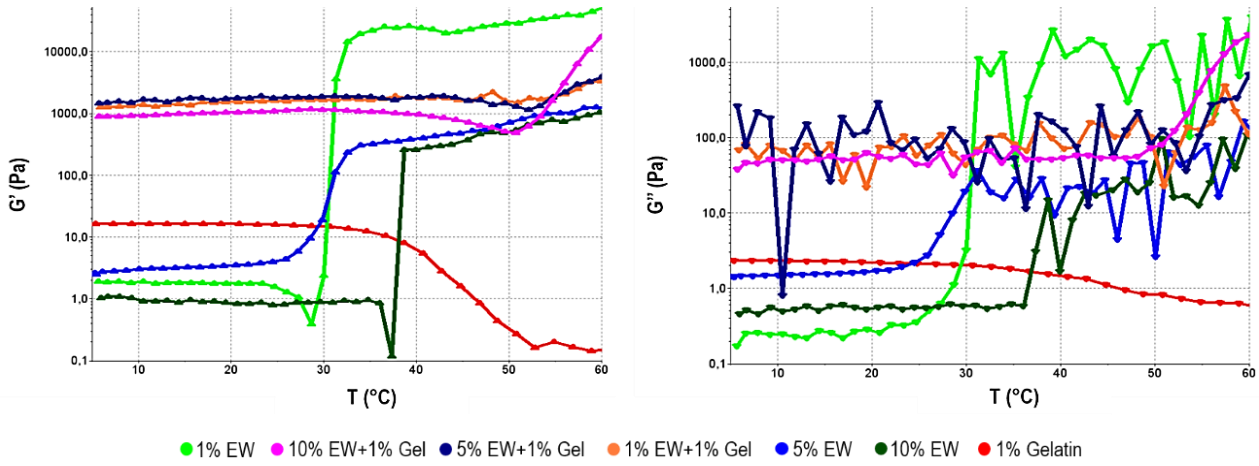
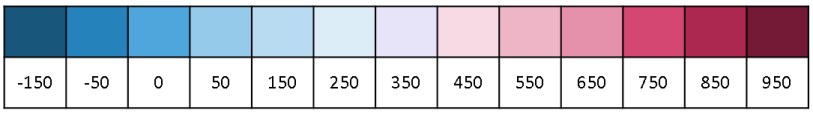


Figure 7: Rheological analysis of the hydrocolloid solutions showing variation of G' (storage modulus) and G'' (loss modulus) with temperature.

4.5. Cell viability and proliferation on 2D coated surfaces

4.5.1. Heat map

The heat map shown in figure 8 allowed us to compare at a glance the cell growth on the different hydrocolloids compared to the control (uncoated well) based on the coloured legend. As it can be easily seen, cells proliferated more on 1% gelatin, 1%EW + 1% gelatin, and 5% EW + 1% gelatin coated surfaces compared to the control.



	Day 1	Day 3	Day 10
1% EW			
5% EW			
10% EW			
1% gel			
1% EW + 1% gel			
5% EW + 1% gel			
10% EW + 1% gel			

Figure 8: Heat map showing cell proliferation. Gelatin is abbreviated as "gel".

4.5.2. alamarBlue® proliferation graphic for 2D surfaces

Cell proliferation was studied between days 1 and 10 in all the colloidal-coated surfaces as well as in control wells. Each colloid was studied in triplicate and the proliferation graph ([Figure 9) was built using the average value minus the alamarBlue® working solution value. The error bars were calculated using the standard error of the mean.

Cell growth was observed on all the colloidal solutions as well as on the control wells. It was observed that 1%gelatin, 1% EW + 1%gelatin, and 5%EW + 1%gelatin colloids have the largest increase in cell proliferation values. Furthermore, cells cultured on the 5%EW + 1%gelatin ciated surface has a significantly higher cell proliferation than cells cultured on the 5%EW and 10%EW + 1%gelatin surfaces.

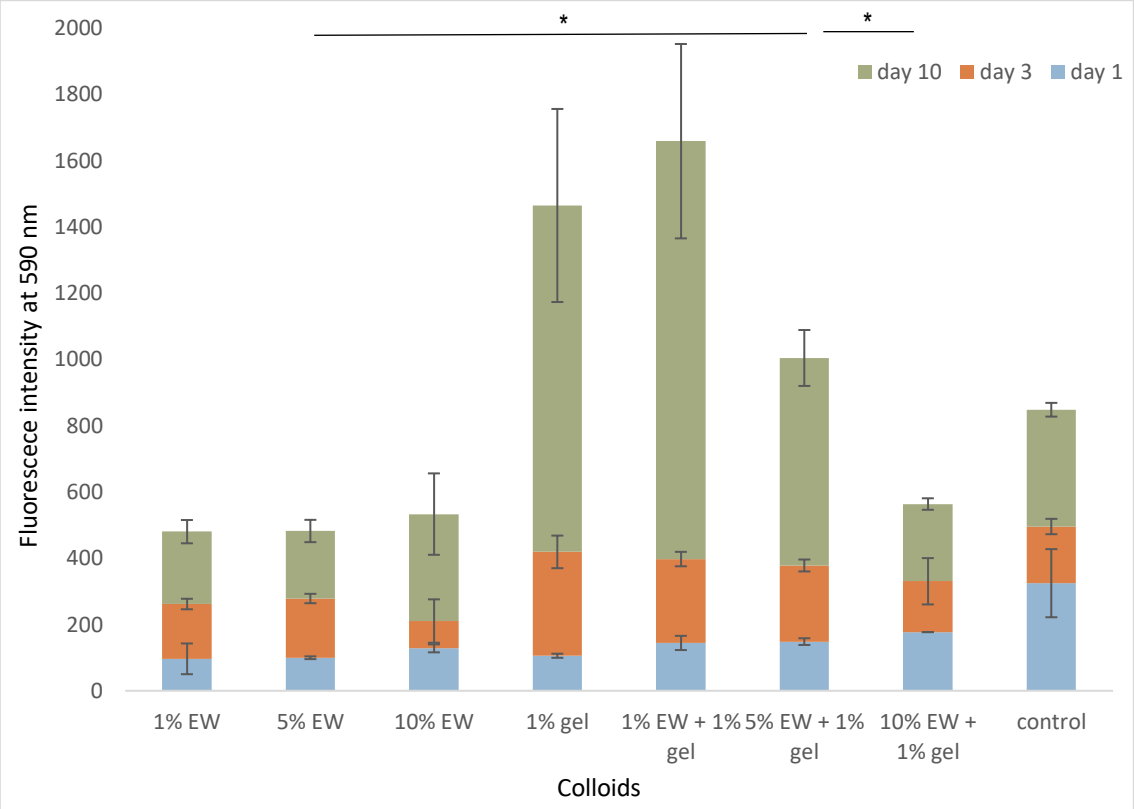


Figure 9: Graph showing cell proliferation comparing fluorescence intensity at 590nm for each colloidal solution. The statistic difference is included (*) showing that there has been growth in 5% EW + 1% gel compared to 5% EW and to 10% EW + 1% gel. Gelatin is abbreviated as “gel”.

4.6. Cell morphology on 2D surfaces

Cell plates were observed and photographed using a phase-contrast light microscope and attached digital camera. Results are presented into a panel showing low confluency and the confluent monolayer the cells ended up forming on all the surfaces ([Figure 10]).

On the control well cells grew as expected, multiplying each day and displaying the typical fibroblast morphology. Confluent monolayers on the control wells showed the typical parallel clusters described in the literature. In the wells coated with the colloidal solutions cells multiplied each day eventually forming a confluent monolayer, as expected. Parallel clusters were seen too, although they were not as regular as the ones formed in the control wells, due to the thicker colloidal solution layer present on the bottom of this wells.

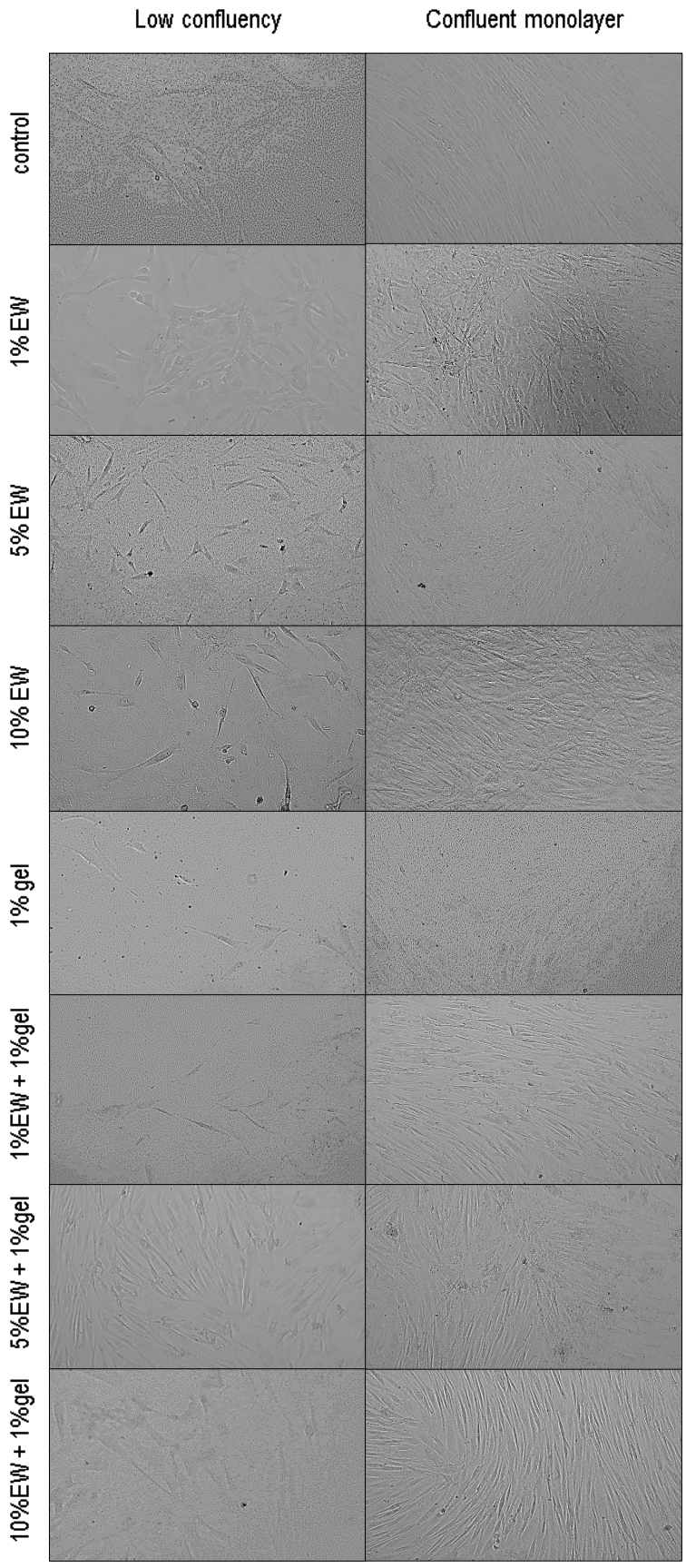


Figure 10: Phase-contrast light microscopy of HDF in each colloidal solution at low confluency and as a confluent monolayer. All the photos were taken at 10X. Gelatin is abbreviated as “gel”.

4.7. Cell viability and proliferation in a 3D miniaturised environment

Based on the results on 2D surfaces, the hydrocolloids 1% EW + 1% gelatin and 5% EW + 1% gelatin were used for microfluidic devices. The hydrocolloids were easily introduced into the central chamber were they were confined, i.e. they did not leak into the large hydrating channels of the device.

Cell proliferation was studied between days 1 and 7 in two microfluidic devices. There were six samples for each hydrocolloid and the proliferation graph ([Figure 11]) was built using the average values. The error bars were calculated using the standard error of the mean.

Cell growth was seen in both hydrogels, although it was observed a slightly higher increase in 1%EW + 1%gelatin hydrogel compared with 5%EW + 1% gelatin, although it was not significant.

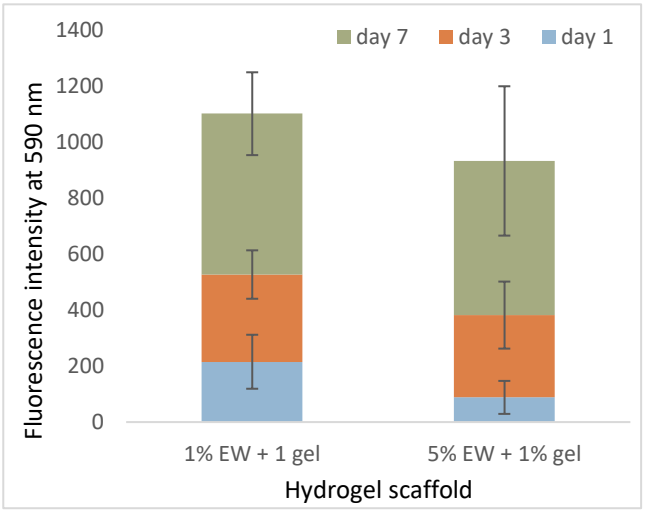


Figure 11: Graph showing cell proliferation comparing fluorescence intensity at 590nm for each hydrocolloid. Gelatin is abbreviated as "gel".

4.8. Cell morphology in a 3D miniaturised environment

Contrast-light microscopy observation showed that cells introduced through the small channels migrated to the central chamber and over time, also migrated to the large hydration channels [Figure 12].

For the devices loaded with 1% EW + 1% gelatin, both flat and rounded cells could be seen in the small channels and the central chambers at day 1 [Figure 12A]. Over time, mostly flat cells could be seen, indicating a predominantly flat substrate. Nevertheless, cells could be seen on different planes at day 7 [Figure 12B], indicating a degree of three-dimensionality.

For the 5% EW + 1% gelatin loaded devices, mostly rounded cells could be seen, as expected to be observed in a 3D matrix [Figure 11A]. Over time, flat cells could also be seen, and they appeared embedded in a 3D matrix. Cells that migrated out into the large hydrating channels were flat as expected and also showed a migrating morphology, with clearly visible lamellipodium [Figure 12B, green arrows].

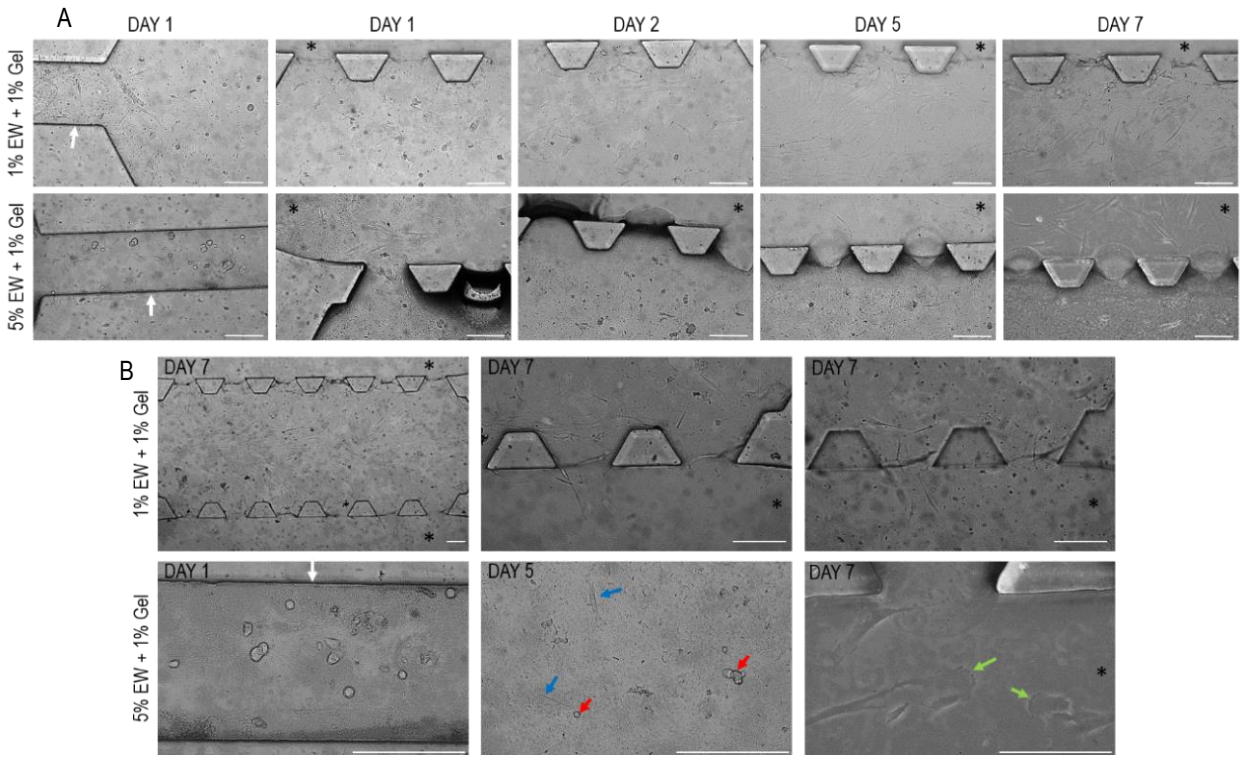


Figure 12: Phase-contrast light microscopy of HDF cultured in 3D miniaturised environments using microfluidic devices loaded with EW and gelatin hydrocolloids. *Indicates large hydrating channels while white arrows point to small channels. (A) Representative images showing cell morphology over time. (B) Additional representative images. For the 1% EW + 1% gelatin panel, the last 2 photos are the exact same view at different planes of observation, showing cells on both. Blue arrows point at cells with a flat morphology whilst red arrows point at rounded cells. Green arrows show lamellipodium. Scale bar is 175 µm. Gelatin is abbreviated as "gel".

4.9. 3D hydrogel scaffolds

Once separated from the mould, 3D hydrogel scaffolds were obtained using 1%EW+1%gelatin and 5%EW + 1% gelatin, their size being ~14 mm diameter and ~4 mm thickness ([Figure 12]). It was observed that both of them were thick and yellowish/orangish in colour depending on the glutaraldehyde concentration used for the cross-linking, being darker at higher concentrations, and the amount of EW in the gel.

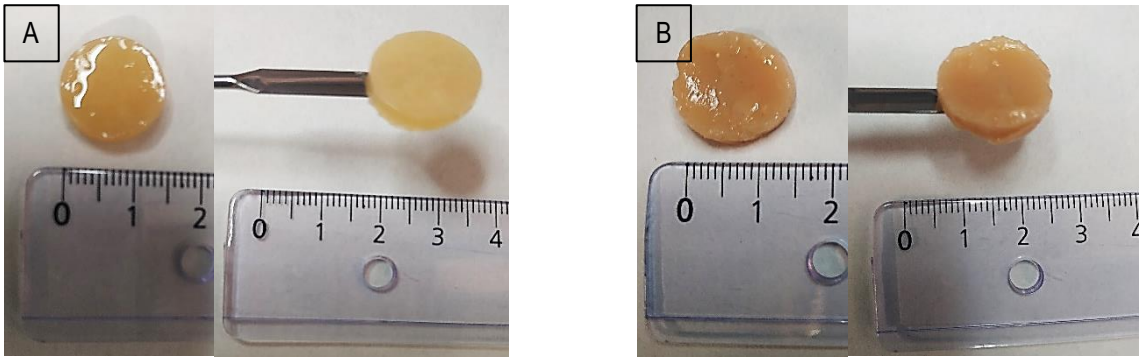


Figure 13: Hydrogel scaffolds of A) 1%EW+1%gelatin cross-linked with 2.5% glutaraldehyde; and B)5%EW + 1%gelatin cross-linked with 5% glutaraldehyde

4.10. SEM of 3D hydrogel scaffolds

Images obtained under SEM ([Figure 14]) show hydrogel scaffolds forming organised and well-defined internal structures. The scaffolds displayed pores that could allow cells to grow and proliferate through them. Also, the 1%EW + 1%gelatin scaffold showed a more compact structure than 5%EW + 1% gelatin, which looked more porous.

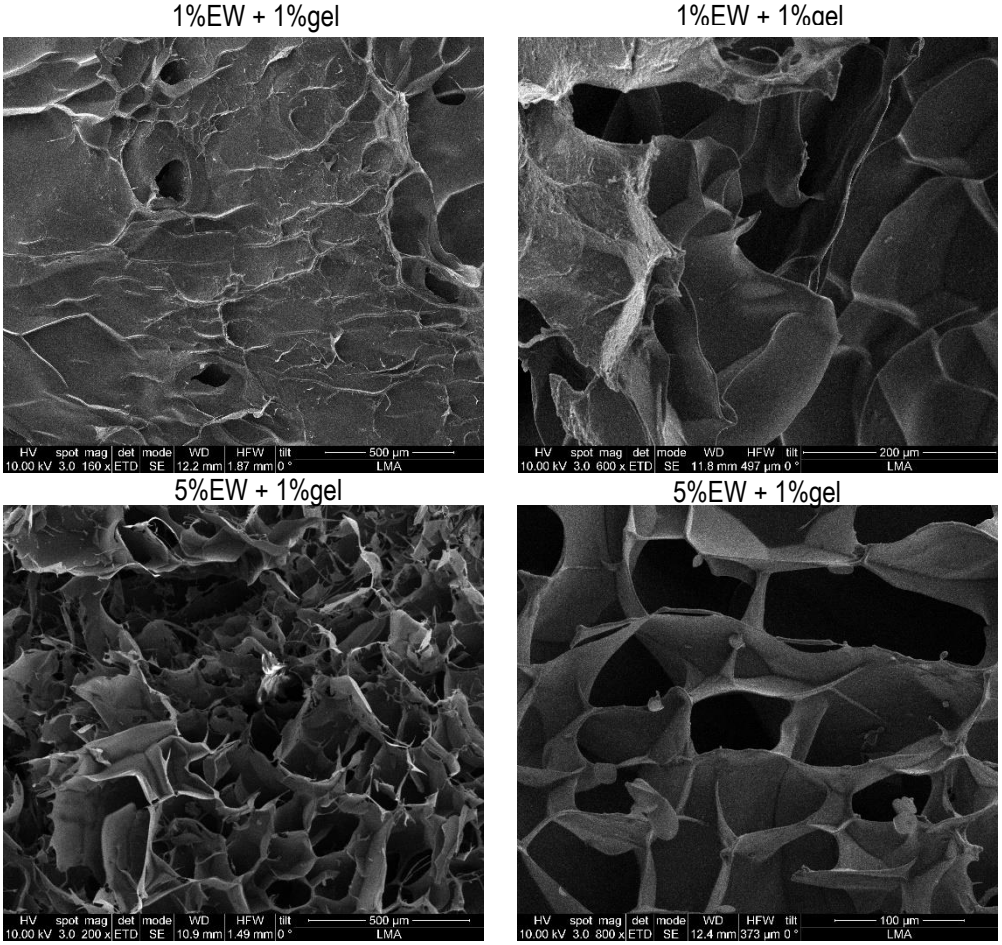


Figure 14: Representative SEM images obtained of 1EW% + 1% gelatin and 5%EW + 1% gelatin scaffolds, both cross-linked with 2,5% and 5% glutaraldehyde, respectively. Gelatin is abbreviated as “gel”.

5. Discussion

The development and fabrication of egg white and gelatin hydrogel-based structures, as well as the study of cell growth and viability on these structures, serve as a crucial first step in biomaterial-based tissue engineering and biomedical applications of these hydrocolloid mixtures, which have already been studied in the food industry (11)(12)(13).

In order to start creating hydrocolloidal solutions coatings and scaffolds, EW was lyophilized into a powder, which was yellowish in colour and very light. This EW powder was mixed with water in different proportions to obtain the colloidal solutions. These solutions were all translucid, yellowish in colour and more viscous with higher EW content, as expected. Also, colloidal solutions are translucid due to the particle size dispersed in the solvent (30). At first glance, there were no observable differences between the solutions with and without gelatin. It was observed that adding EW alkalinises the solution, so it had to be neutralized using an acid. Also, for the colloidal solutions rheological characterisation was conducted. Rheological

analysis showed a predominantly elastic behaviour over a viscous one as the modulus of storage G' was higher than the loss modulus G'' (31). The mixed hydrocolloids showed an increased viscosity and gel behaviour over the individual hydrocolloids. Due to the difference in behaviour with increasing temperature between the individual hydrocolloids, results suggested that temperature could be used to fine-tune the viscosity of the resulting gels. Future rheology work would involve the use of a different sensor to eliminate the noise for G'', increasing the temperature beyond 60 °C, and the study of other rheological parameters like complex viscosity or the phase angle.

Hydrogels coatings were made, and cells were seeded on them. In order to analyse cell growth and viability, two factors were studied: cell proliferation and cell morphology. Cell growth and proliferation was successful in every type of colloidal coating, as the alamarBlue® assay showed an increase in the number of with culture time. It was observed that 1% EW + 1% gel and 5% EW + 1% gel were the surfaces with a higher cell growth rate, compared to the other ones. As a different representation of the results, the heat map also showed an increase in cell growth on these two solutions. Cells cultured on the 1% gelatin surface showed increased proliferation compared to the control, which was expected as this coating is often used to maximise cell culture (29), and so this surface was also used as control.

For the cell morphology analysis, it was be studied whether cells maintained their typical shape on the different colloidal-coated surfaces and were able to eventually form a monolayer. The typical fibroblast morphology is elongated, where one elliptical nucleus with multiple nucleoli are noted as well as a clearly visible endoplasmic reticulum. Also, at high confluency cells arrange forming a confluent monolayer with parallel clusters (29). Cells grew correctly over time showing they were viable and able to grow and conform a monolayer on 2D structures based on the hydrocolloids. Further work on 2D surfaces would involve investigation of other cellular events like, for example, the migration. It would also be interesting to study the different surfaces in terms of topography and wettability, to better understand the differences in cell proliferation observed in this study, as it has been shown that the material properties of 2D surfaces affect cell behaviour (32)(33).

The results obtained on 2D surfaces pointed to the fact that the most efficient hydrocolloids for cell growth and proliferation were 1% EW + 1% gelatin and 5% EW + 1% gelatin. Therefore, these two were chosen for carrying out the rest of the project.

As already explained, the chosen hydrocolloids were easily introduced into the central chamber of the microfluidic devices used in this study. Moreover, alamarBlue® assay conducted in the microfluidic devices showed cell growth over time in both hydrogels. The results on the microfluidic devices suggested that hydrocolloids of EW and gelatin could be used for cell studies using these miniaturised cell culture platforms. Interestingly, a 3D ambient can be created inside the devices, specially with the 5%EW + 1% gelatin hydrocolloid. As with the 2D surfaces, further work on using these hydrocolloids in microfluidic devices would involve the study of cellular behaviour like the already mentioned cell migration.

Hydrogel scaffolds were fabricated with 1% EW + 1% gelatin and 5% EW + 1% gelatin solutions, using glutaraldehyde as a cross-linking agent. Thermal gelation correctly occurred correctly in both solutions and 3D hydrogels were obtained. Under electron microscopy the hydrogels presented an orderly porous structure, specially for the 5%EW + 1%gelatin 3D hydrogel, which could be due to the higher amount of protein in these scaffolds. Porosity is a desired feature for 3D scaffolds as it allows cellular infiltration as well as diffusion of nutrients and waste products (34). Further work would involve thorough material characterisation as well as *in vitro* cell work using these 3D hydrogels.

6. Conclusion

We can conclude that this project´s objective was accomplished. Hydrocolloids of egg white and gelatin were successfully used to create a platform for hydrogel-based tissue engineering. Properties of mixed EW and gelatin hydrocolloids were determined, and they allowed the establishment of cell cultures on both 2D surfaces and microfluidic devices, as well as the fabrication of 3D hydrogel scaffolds.

These results show that it is feasible to use hydrocolloids of EW and gelatin for hydrogel-based tissue engineering, which has numerous applications, in the biomedical field. This work serves as a first step for future research and experimentation in the field of tissue engineering.

7. Future work

As mentioned before, this work serves as a first step for future experiments using these hydrocolloids in the field of tissue engineering. It has been shown that cells are able to grow and proliferate on the 2D hydrocolloidal coatings and miniaturised 3D environments designed in this project. As a different approach, tumoral cells could be cultivated on them, which would allow us to study their feasibility to build cancer models. 3D hydrogel structure could be further analysed using a multiphoton microscope or by TAMRA-labelling and light sheet microscopy analysis. In addition, more material properties of the 3D hydrogel scaffolds could be studied in different experiments like swelling or degradation rate assays.

All of this could contribute to the development of biological substitutes for the treatment and improvement of organ and tissue functionality in the human body, as well as building 3D tissue models for the study of cellular phenomena.

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Abbreviations

2D: bidimensional

3D: tridimensional

ECM: extracellular matrix

EW: egg white

Gel: gelatin

HCL: hydrochloric acid

HDF: human dermal fibroblasts

H_2O: water

dH_2O: distilled water

NaOH: sodium hydroxide

pnHDF: primary normal human dermal fibroblasts

SEM: Scanning Electron Microscopy