Master thesis : Fabrication of free-standing casein devices with micro- and nanostructured regular and bioimprinted surface features

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Characterization of casein-based films for cell-culture and medical implants

Master Thesis conducted by

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in order to obtain the degree of Master in Biomedical Engineering

under the supervision of

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Dr. Dipl.-Ing. Volker Nock (New Zealand)

University of Liège
Faculty of Applied Sciences

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Abstract

Master Thesis

Characterization of casein-based films for cell-culture and medical implants

by F. de DECKER

The objective of this thesis was to optimize casein-based biodegradable films for use as cell culture substrates and medical implants. These films were patterned with 3D imprints of cells to influence cell phenotype. Secondary cells align on surface patterns and dissolve the material in the process. In order to find the best films, characterization of these films was made according to their biocompatibility, flexibility, stability and high resolution of imprints.

More specifically, the characterization of different casein-based films was studied as a function of protein and glycerol concentration as well as the type and the concentration of crosslinking reagents. Films were crosslinked with glutaraldehyde, formaldehyde, citric acid and transglutaminase.

Mechanical properties such as stiffness and glass transition were characterized using Dynamic Mechanical Analysis (DMA). Spectrophotometry was used to measure the transmittance of the films and an epifluorescence microscope at 595 nm was used to analyze the film autofluorescence. Contact angle, degradation time and water uptake ratio were also investigated in DI-water and in media at 37.5°C in order to mimic the cell environment.

An increase of glycerol concentration level increased the transmittance of the film. However, an increase of casein concentration level decreased the transmittance. On the other hand, glutaraldehyde and citric acid films had an orange-brown color and did not transmit light under 400 nm.

Casein-based films were not enough autofluorescent to influence cell analysis using fluorescent dyes or labels except citric acid and glutaraldehyde-crosslinked films. Indeed, the two latter films had saturated fluorophores with an exposure time of 2 seconds.

An unexpected result was observed regarding to contact angle. TG-crosslinked film had the highest contact angle and was the only cell-friendly film. Contact angle of the film did not influence the cell adhesion.

Degradation time of the films varied according to the crosslinker concentration. Higher the crosslinker concentration level was, higher the degradation time was. In addition, degradation time of films immersed in media was longer than films immersed in DI-water.

Swelling of the films decreased with the increase of crosslinker concentration level. Formaldehyde and citric acid films had a low water uptake ratio. TG-crosslinked casein-based film had a very high water uptake ratio in DI-water but not in media. Diameter expansion and patterning expansion of crosslinked films were also investigated. In a logical way, their expansion was lower in media.
An optimal film for use as cell-culture substrate could be deducted from these results. Casein-based film with 10 U/g of transglutaminase was transparent, slightly fluorescent, biocompatible and did not expand much in media (about 30%). All of these characteristics are favorable for this application. Indeed, standard microscope can be used for cell analysis if the material is transparent. A biocompatible material induces a good cells adhesion. If the material does not absorb a lot of water, bioimprints are similar to the cultured cells throughout the process and cells might differentiate.

Another aspect of this project was aiming to develop 3D structures of casein by mixing casein solution with gelatin to add gelation properties. Gelatin/casein films were transparent, slightly autofluorescent and did not absorb a lot of water. They are promising for use as cell-culture substrates and medical implants.
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5.6 Degradation time (in minute) of crosslinked casein-based films in media at 37.5°C.
Introduction

This work is part of a major project which started three years ago in Electrical and Computer Engineering Department, University of Canterbury (UC), Christchurch, New Zealand. The goal of this major project has been to develop casein-based biodegradable films for use as cell-culture substrates and in medical implants. More specifically, it consists of developing ways to pattern the surface of casein films with 3D imprints of cells, called bioimprints, to influence cell phenotype. Secondary cells align on surface patterns and dissolve the material in the process.

This project was sequenced in three main steps:

1. Material optimization: obtain a material with suitable characteristics depending on its use, i.e. material needs to be biocompatible and transparent to be used as cell-culture substrate, and material needs to be flexible and stable to be used in medical implants.

2. Patterning process: determine a high resolution replication of regular features and bioimprints on casein-based biodegradable materials which are based on casein using micro- and nanopatterning techniques.

3. Cell-culture: analyse the cell behaviour on patterned, biodegradable materials.

These steps has been investigated by the PhD student, Azadeh Hashemi, for the last three years. At this stage, cells did not adhere to the films. Consequently, characterization of the films could help to understand cell adhesion. In addition, when a new material is developed, it is also important to know all its properties. Different test on different samples of different concentration levels were analyzed in order to understand the influence of each compound of the casein-based films and find the best films for cell-culture substrate and medical implant applications.

This master thesis will investigate the characterization of different casein-based films. A literature review and an overview of what has been already done in the project (i.e. casein optimization and microfabrication, patterning process and cell-culture) will be presented in Chapter 1. In Chapter 2, film preparation as well as the first observations will be described. Mechanical properties such as stiffness, optical properties such as transmittance and autofluorescence and physical properties such as contact angle, water uptake ratio and degradation time will be presented in Chapter 3, 4 and 5, respectively. In these chapters, the experimental setup for each test will be first described. Then, the results will be presented and discussed. Finally, conclusion and future works will be discussed in Chapter 6.
Publications have (will) come out of this work:

- Journal publication: Journal of Vacuum Science and Technology (under review).

- Conference presentation: The 61st International Conference on Electron, ion, and photon beam technology and nanofabrication, Orlando, Florida, United-States.
Chapter 1

Background

1.1 Literature review

1.1.1 Medical implant

Medical implants have been advanced through developments in science and engineering for many years. They are defined as biological (skin, bone, tissue) or artificial (metal, plastic, ceramic, composite) materials inserted or grafted into the human body. They may deliver medication, replace missing parts of the body, monitor body functions and/or provide support. They also may be permanent or removed once they are no longer needed [1]. Many implantable medical devices such as pacemaker, cochlear implant, artificial hip, and real-time blood pressure sensors have been developed and improved over the years [2]. Still, implants include surgical risks during placement or removal. An infection may occur or the implant may fail. Structural design and material of the implant need to be chosen to have an appropriate biological host response. This characteristic is named biocompatibility [2, 3]. When the medical device is implanted in the human body, the surrounding environment immediately responds to the material itself or to micro-organisms on the surface of the device. In order to have a positive interaction with an implanted device, the surface of the material usually undergoes chemical or physical modifications. Biodegradable devices prevent these risks [4]. Synthetic biodegradable polymers are currently used or under investigation. The largest and longest use of these polymers in medical applications is for suturing or internal fixations for fracture such as rods, plates, screws [5, 6].

1.1.2 Cell-culture

Nowadays, cell-culture has become a major tool in life science. It consists of different techniques used to grow cells outside of their natural environment for the purpose of experiments. These types of experiments are called in vitro experiments. In 1907, R.G. Harrison presented the first method of cell-culture for embryonic nerve cells of frogs [7]. Cell-culture permits live cells to be observed in favorable and controlled conditions. The most common substrates for supporting cell growth are made from polystyrene (PS: plastic) or glass (more expensive) and take the form of a flat two-dimensional surface. In cell-culture, most of the cells have a substrate attachment dependence. Substrates usually undergo surface modification to ease cell adhesion. For instance,
PS substrates have good optical clarity, are easy to mould, and have the ability of being sterilized by irradiation. However, PS is hydrophobic, which makes cell attachment difficult. Hence, PS is treated by corona discharge under atmospheric condition or gas plasma under vacuum to obtain a more hydrophilic surface. Indeed, plasma membranes have a negative charge on their surface [8]. Cell adherence is tailored by specific surface receptors for molecules localized in the extracellular matrix. Stiffness (stress fibers), porosity, rugosity/roughness and sterilization method of the material are properties that can also influence the cell behavior regarding the substrate [9]. Moreover, the presentation of a cell to a two-dimensional glass or PS substrate is not an accurate representation of the extracellular matrix found in native tissue. Obtained results in vitro are not always easy to interpret in vivo where parameters are more numerous. Three-dimensional relations are lost, as well as systemic components related to homeostasis (nervous and endocrine systems). The role of a normal cell from division, through proliferation to migration and apoptosis, is an accurately controlled series of events that inherently rely on the principles of spatial and temporal organization [10]. In vivo, cells grow on top of other cells and receive signals from the cells and extracellular matrix they adhere to. Cell adhesion is influenced by their surroundings, extra-cellular matrix and the substrate.

1.1.3 Bioimprint

Bioimprint was first developed in 2006 and defined as a permanent capture of replica impressions of biological cells into a polymer at nanometer resolution [11]. Cells cultured on surfaces with cell imprints have been shown to behave differently than cells cultured on flat surface. Regarding medical implants, previous studies on the effects of patterned and imprinted substrates on cell growth have demonstrated that surface modification may lead to faster and more extensive implant integration and higher long-term stability [4, 12]. To this date, high resolution surface patterning for biomedical application has been confined to conventional tissue engineering plastics such as PS and specific metals used for medical implants [13]. In brief, surface topography is increasingly recognized as an important parameter, which can influence the development and function of biological tissue (phenotype). Cells, i.e. Human Nasal Chondrocytes, seeded on bioimprinted polymer surfaces exhibited distinct behaviour compared to flat surfaces regarding their attachment and proliferation.

The process of 3D printing has been extensively developed for nonbiodegradable materials (polydimethylsiloxane, PS, methacrylate) by Volker Nock, Maan Alkaisi from the University of Canterbury and Ali Azam from the University of Otago, Christchurch, New Zealand [11, 14–17]. Fabrication of patterned casein-based films as cell-culture substrates, has been done for the first time by Azadeh Hashemi, Volker Nock, Maan Alkaisi and Ali Azam in 2013 under the project [18].

1.1.4 Biodegradable materials

Currently, the main biodegradable materials used in biomedical field are natural polymers such as collagen, gelatin, chitosan or synthetic polymers such as poly-L-Lactic acid (PLA). Natural materials are more similar to materials in the body. However, they have a higher disease
Transmission rate. In addition, ceramics such as tricalcium phosphate and hydroxapatite (HA) are presently used in orthopedic surgery, but they have a poor fracture toughness due to their brittleness. Some metal alloys are also used as biodegradable materials, but they can have a high rate of corrosion which influences badly their biodegradation [6,13]. In this work, we introduce casein, a highly promising biopolymer for medical implants and cell-culture substrates.

1.1.4.1 Casein

Casein from Latin word ‘caseus’, which means ‘cheese’ is the major protein of cow’s skimmed milk, i.e 80% of the proteins in mammalian milk and 20%-45% in human milk [19]. It is made up of four phosphoproteins: α-casein (α s1 and α s2), β-casein, γ-casein and κ-casein. Since milk is mainly made up of water, casein is considered as a water soluble protein and has the ability to be patterned accurately.

Casein-based polymers were first introduced in the early 20th century. They are both renewable and biodegradable [20]. Caseins have shown to be useful in adhesives, micro-encapsulation, textile, food industries and pharmaceuticals [21]. Microscale devices made of casein have since been proposed for use as tissue engineering scaffolds and degradable, stand-alone orthopaedic implants [22]. Glutaraldehyde-crosslinked casein conduits (GCCs) have recently been shown to promote regeneration of peripheral nerve after an injury in adult rats [23]. Different techniques have been proposed for the fabrication of casein-based devices via spin-coating, solution-casting and spray-coating [24]. Strong mechanical microstructure and no toxicity make casein a suitable material for implantation, but acute inflammatory reactions of the surrounding tissue remain a problem [23].

1.2 Previous work at UC

In this section, the work done by the PhD student, Azadeh Hashemi, for the last three years is presented. As mentioned before, casein-based polymers needed to be optimized in order to meet the requirements for use in potential biomedical applications such as cell-culture substrates and medical implants. Additionally, the patterning process has been studied. A method for replication of micro- and nanoscale regular features (square, triangle, circle, cross), and biological cells was developed. Finally, secondary cell-culture on patterned films has been performed to analyze cell response and morphology according to film composition and patterning. In the following sections, all these concepts are explained or illustrated.

1.2.1 Casein optimization

Protein-based materials are usually made of different additives. In this project, casein films were made up of casein powder, buffer, plasticizer and crosslinking agent to tailor their properties and improve their formation. The aim of the casein optimization was to find the best composition of the films according to their flexibility, stability, biocompatibility (positive biological response), optical and mechanical properties and suitability for high-resolution patterning. In
order to perform this optimization, various films were made with different concentration levels of casein, plasticizer and crosslinker using two different buffers. The results of the patterning process applied to these films, as well as the cell-culture are discussed in Section 1.2.2 and 1.2.3, respectively.

Considering that casein solubility is pH dependent, basic elements needed to be added or a buffer needed to be used to make casein more soluble [25]. Some films were made by dissolving casein powder in sodium hydroxide (NaOH) to obtain caseinate-based films [24]. Other films were made with disodium hydrogen phosphate (Na$_2$HPO$_4$) used as a buffer by adjusting the pH to 7 [23]. A plasticizer, such as glycerol, was used to obtain a plastic film. It reduces polymer-polymer chain secondary bonding and increases the mobility of the macromolecules due to the augmentation of the free volume. As mentioned before, casein is considered a water-soluble protein and dissolves in water in a few hours. Biodegradability can be tailored by crosslinking. The role of a crosslinker agent is to link polymer chains, restricting the ability of movement, to increase the stability of the polymer against thermal and mechanical denaturation [20] and obtain a degradation time sufficient for cell-culture (days-weeks). In this project, crosslinking has been done by a chemical reaction between crosslinker agents and casein. Four different crosslinking agents were studied: glutaraldehyde [23], formaldehyde, citric acid and transglutaminase (TG). The minimum concentration able to crosslink the films was determined and used.

Films were made of 5, 10, 15 and 20% of casein powder in 0.2% NaOH or 3% Na$_2$HPO$_4$ mixed with 0, 5, 15 and 25% glycerol. Glutaraldehyde, formaldehyde and citric acid were mixed with casein solution with 0.5, 1 and 2% concentrations. Transglutaminase was mixed with casein solution with concentration of 10 U/g of casein protein. One unit of transglutaminase activity is defined as the amount of enzyme catalysing the formation of 1 $\mu$mol of hydroxamate per minute from N-Carbobenzoxy-Glutaminyglycine (N-CBZ-Gln-Gly) and hydroxylamine at pH 6.0 at 37 degrees [26].

### 1.2.2 Patterning process

Surface topography is increasingly recognized as an important parameter which can influence cell phenotype [12]. A method used to print cells on the material surface has been developed. It aimed to promote adhesion of these cells on the surface, as well as improve biocompatibility and growth. In other words, it allows to mimic the natural cell environment.

As a first step, regular features e.g. lines and dots arrays, were transferred on different casein-based films to evaluate the accuracy of the transferred patterns. It was easier to measure the accuracy of the replication of regular features than biomimetic features. The process consists of different steps as fabrication of intermediate moulds with geometric patterns by photolithography and soft lithography (photoresist, mask, liquid-casting) as detailed in Ref. [18]. Features of the replication were compared to the original features by optical microscopy and Atomic Force Microscopy (AFM), as shown in Figure 1.1.

From the results obtained, the best composition of casein film to optimize the mechanical
stability, the flexibility and the pattern resolution was 15% casein in 0.2% NaOH solution mixed with 10% glycerol and cross-linked by addition of 2% glutaraldehyde [18]. However, the pattern resolution was limited and details were lost, as shown in Figure 1.1. Actually, this is due to the fact that PDMS is hydrophobic, with a measured contact angle of around 110°. Casein did not penetrate well into the microscale features on PDMS moulds. Consequently, further investigation was done to improve this. Additional steps were added to the replication process. PDMS moulds were plasma-treated for 180 seconds in 30 W oxygen plasma (K1050X Plasma Etcher, Quorum Emitech). This step increased the surface energies and the PDMS moulds became more hydrophilic. Nevertheless, the effect of oxygen plasma decayed with time due to impurities in the air. The contact angle of plasma-treated PDMS immediately after treatment was reduced to less than 10°, but it increases to more than 50° after 30 minutes. Therefore, PDMS moulds were immersed in a polyvinylpyrrolidone (PVP) solution for 60 seconds prior to liquid casting casein onto the moulds. Moulds became more permanently hydrophilic than if they were only plasma-treated [27]. A 22.2% PVP solution was prepared by dissolving PVP in DI-water by stirring. After treating PDMS with PVP for 1 minute, it was taken out of the solution, rinsed thoroughly with DI-water and dried using a nitrogen gun. The contact angle of PDMS after being plasma/PVP treated decreases to less than 10° and it remains the same even after 1 hour.

Secondly, a similar method to replicate live cells onto the film, named bioimprint, was developed. It was performed in different steps: culture and fixation of C2C12 mouse myoblasts on patterned microscope slides, replication onto PDMS and casein using liquid casting and drying [18]. C2C12 myoblast cell line was chosen as it is a useful tool to study differentiation (myoblast and osteoblast), protein expression and mechanistic pathways [28, 29]. The cells were
obtained from the University of Otago, Christchurch School of Medicine, as frozen vials of cells with passage number 32. Moreover, plasma and PVP-treatment of the PDMS mould were done. Microscope slides were patterned with a set of three grids to compare imprinted features with the original features at different steps of the replication. Results were very conclusive, as presented in Figure 1.2. It can be seen that plasma/PVP treating PDMS moulds improved the resolution of the features on the final imprint compared to no treatment and results obtained using the previous process [18]. These features can be directly compared to the original cells on the glass. As shown on the graph of the cross section of the cell across the drawn line in the AFM image at different stage of bioimprinting, surface topography of the original cell (green line) is similar to the surface topography of the replicated cell onto casein film with PVP treatment (black line).

Figure 1.2: Replication of bioimprint onto casein: (a) AFM image of a fixed cell on the glass slide, (b) AFM image of inverted imprint on PDMS, (c) AFM image of imprint onto casein without PVP treatment of PDMS, (d) AFM image of imprint onto casein with PVP treatment of PDMS, and (e) graph of the cross-section of the cell across the drawn line in the AFM image at different stage of bioimprinting. Adapted from [30].

1.2.3 Secondary cell-culture

In order to find the best composition for casein based films used as cell-culture substrate or medical implants, it was very important to assess cell behaviour with respect to the material in terms of biocompatibility and cell adhesion.

Secondary cell-culture of C2C12 mouse muscle cells on different crosslinked and patterned casein films was studied. As mentioned before, films need to be crosslinked to increase their degradation time in water or cell-culture media. Four different crosslinking agents were studied: glutaraldehyde [23], formaldehyde, citric acid and transglutaminase (TG).

Films were first UV sterilized for 30 minutes before placing cells and then incubated for 24 hours. Results for glutaraldehyde-crosslinked films and citric acid-crosslinked films with 15% casein and 15% glycerol are shown in Figure 1.3.
1.2.3.1 Glutaraldehyde

Glutaraldehyde is from the aldehyde family and has been used as a crosslinker for casein in medical applications [23]. It contains two reactive functional groups which react with the same amino-acid chain. This homobifunctional compound links covalently to the amine groups of lysine in the protein molecules creating a more stable structure [31]. Cross-linking was efficient. However, glutaraldehyde cross-linking had some negative points. When glutaraldehyde-crosslinked casein-based films were immersed in water or cell-culture media they were observed to expand, leading to a distortion of features. In addition, media became darker, meaning that glutaraldehyde killed the cells. Indeed glutaraldehyde is considered a cell fixative, which kills cells by crosslinking their proteins at high concentrations. The minimum concentration to crosslink casein-based film was high enough to kill the cells. Washing the films with DI-water and media was tried to remove the excess glutaraldehyde in the films, but it still lead to cell death. A solution would be using another crosslinker.

1.2.3.2 Formaldehyde

Formaldehyde cross-linker, similar to glutaraldehyde, was also investigated. It is the simplest of aldehydes and reacts with N-terminal amino groups and side chains of cysteine, histidine, lysine, tryptophan and arginine [32]. It is another crosslinker used for crosslinking proteins such as casein [33]. The results were similar to glutaraldehyde except in film expansion. Formaldehyde-crosslinked films slightly expanded. Formaldehyde would leach out of the films in cell-culture and kill the cells by crosslinking of cell proteins.

1.2.3.3 Citric acid

Citric acid is inexpensive and a non-toxic chemical which has been used in textile applications to improve the performance properties of proteins [34]. Citric acid can act as a plasticizer or a crosslinker in function of the pH [35]. The size of the casein sample changed slightly and films were dissolved in few days or weeks. However, patterns onto the surface of the films disappeared in a few minutes. Additionally, cells did not adhere to the crosslinked casein-based films as expected. They grew underneath the films on the PS surface of the cell-culture flask. In order to discourage the cells from adhering to PS and make them adhere to the films, the surface of the PS was covered with a thin layer of poly-2-hydroxyethyl methacrylate (PolyHEMA) and films were placed on the liquid polyHEMA. PolyHEMA is a water-swellable polymer. The plates were then placed on a hot plate at 60°C for 30 minutes so that polyHEMA would solidify and casein films would stick to the PS dish. Cells were then seeded on the films, but they still did not adhere to the films. Therefore, several ways to make the surface of the films more cell friendly were investigated, such as coating of the casein film with fibronectin and collagen. Fibronectin and collagen are constituents of the extracellular matrix which ensures cell adherence [36]. Films were treated with collagen with two different methods, namely the mixing collagen with the solution before liquid casting with concentration of 75 µg per gram of casein or coating the surface of crosslinked films with 5 µg/cm². Collagen I from rat tail (A10483-01, Gibco) was diluted to 100 µg/ml in 1 × Phosphate Buffer Saline (PBS) from 3 mg/ml. 1 × PBS was prepared by dissolving 1 PBS tablet (003002, Life Technologies) in 100 ml DI-water. Films made with
both methods were rinsed five times with media, before being used as cell-culture substrates, to remove the acetic acid originally existing in the collagen solution from the films as much as possible. However, cells were still not able to adhere reliably to these films. In order to treat casein films with fibronectin, 1 ml of PBS was added to 1 tube of fibronectin. Then, 100 µl of fibronectin solution was poured onto a casein film with 10 cm$^2$ surface area. Fibronectin solution was left on the films for 30 minutes. It was removed before seeding cells on the films. Cells were observed under optical microscope at time zero and after 24 hours. Cells do not appear to adhere to the films. In order to obtain a higher hydrophilicity by increasing the surface energies, plasma treatment of surface of the casein film was also done. Unfortunately, these tests were not conclusive and the cells did not adhere to the casein-based film.

1.2.3.4 Transglutaminase

Finally, TG-crosslinked films were studied. TG is an enzyme catalyzed protein and selectively mediates the chemical reaction between glutamine and lysine residues on adjacent proteins, thus providing covalent amide bonds that serve to reinforce the three-dimensional matrix [37]. The process of crosslinking was based on protocol and TG did not crosslink the film and was discarded. The characterization of different casein film compositions could help understand why cell do not adhere to the casein film and may allow one to find a solution.

In the same time, another process of TG crosslinking was performed. Previous method was insufficiently complete to get good results. These new tests showed that the concentration of TG was sufficient to crosslink the films and was not toxic for the cells. Actually, cells adhere to the TG-crosslinked casein-based films as shown in Figure 1.4. Consequently, this type of crosslinking is very promising for this project and further investigations will be done. The characterization of different casein film composition is useful to understand why cells adhere on TG-crosslinked casein based films and not on the others casein-based films.
This thesis will investigate the characterization of different casein films without patterns to focus on the material properties. Mechanical properties such as stiffness and glass transition, optical properties such as the transparency and autofluorescence and physical properties such as the contact angle, the water uptake ratio and the degradation time will be studied in Chapter 2.
Chapter 2

Film preparation

Film preparation is a crucial part of this research project in order to support the outcomes. First, materials used are presented. The process of film preparation including crosslinking is determined, as well as each composition of casein films. Then, gelatin/casein film preparation are introduced. Finally, first observations of the films are discussed.

2.1 Materials

Casein films were made up of different compounds, as mentioned in Section 1.2.1. Casein powder from bovine milk (C7078-500G), gelatin from porcine skin (G2500-500G) and sodium hydroxide pellets (NaOH, S5881-500G) were supplied by Sigma-Aldrich, as well as glutaraldehyde (G5882-50ML, 25 % in H$_2$O) and formaldehyde (252549-1L, 37 wt. % in H$_2$O) solutions and Polydimethylsiloxane (PDMS, Sylgard 184 silicone elastomer base and curing agent) used for patterning. Glycerol was supplied by ThermoFisher Scientific. Citric acid was purchased as a powder from a local supermarket. Transglutaminase (TG, 100U/g) was a kind gift from Ajinomoto Co Inc., Japan. Cell-culture media was composed of Dulbecco’s Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Fungizone and Penicillin/Streptomycin (P/S) solution (P/S) supplied by Life Technologies. Phosphate Buffered Saline (PBS, 003002) was also purchased from Life Technologies. Lastly, disodium hydrogen phosphate (Na$_2$HPO$_4$, 5234437) provided by Merck was used as second buffer.

2.2 Composition

In order to study the influence of casein and plasticizer concentration, sixteen films have been made based on several concentration levels in 0.2% NaOH. Casein concentration ranged from 5% to 20% with a step of 5%. These low casein concentration levels allow one to obtain not too hard casein-based films. Glycerol concentration was 0, 5, 15 and 25%. A higher concentration level is not needed because the film would be too soft or liquid. On the other hand, four films have also been made with 3% Na$_2$HPO$_4$ in order to assess the influence of buffer type. The composition of these non-crosslinked (NC) casein-based films is given in Table 2.1.

Then, in order to study crosslinking, several crosslinkers characterized by specific concentra-
Crosslinked films were made of 15% casein in 0.2% NaOH and 15% glycerol. They were crosslinked by 0.5, 1 and 2% glutaraldehyde, formaldehyde and citric acid and 1 U/g, 5 U/g and 10 U/g transglutaminase. As a reminder, one unit of transglutaminase activity is defined as the amount of enzyme catalysing the formation of 1 µmol of hydroxamate per minute from N-Carbobenzoxy-Glutaminylglycine and hydroxylamine at pH 6.0 at 37 degrees [26]. The composition of each crosslinked (C) casein-based films is given in Table 2.2.

<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th>Glycerol</th>
<th>NaOH</th>
<th>Na$_2$HPO$_4$</th>
</tr>
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<tbody>
<tr>
<td>NC1</td>
<td>5%</td>
<td>0%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC2</td>
<td>5%</td>
<td>5%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC3</td>
<td>5%</td>
<td>15%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC4</td>
<td>5%</td>
<td>25%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC5</td>
<td>10%</td>
<td>0%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC6</td>
<td>10%</td>
<td>0%</td>
<td>-</td>
<td>3%</td>
</tr>
<tr>
<td>NC7</td>
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<td>5%</td>
<td>0.2%</td>
<td>-</td>
</tr>
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<td>NC8</td>
<td>10%</td>
<td>15%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC9</td>
<td>10%</td>
<td>15%</td>
<td>-</td>
<td>3%</td>
</tr>
<tr>
<td>NC10</td>
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<td>-</td>
</tr>
<tr>
<td>NC11</td>
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<td>0%</td>
<td>0.2%</td>
<td>-</td>
</tr>
<tr>
<td>NC12</td>
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<td>0%</td>
<td>-</td>
<td>3%</td>
</tr>
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<td>5%</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>NC15</td>
<td>15%</td>
<td>15%</td>
<td>-</td>
<td>3%</td>
</tr>
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Table 2.1: Composition of non-crosslinked (NC) casein-based films. Each concentration of casein, glycerol, NaOH and Na$_2$HPO$_4$ is expressed in percentage by weight (wt%).
<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th>Glycerol</th>
<th>NaOH</th>
<th>Glutaraldehyde</th>
<th>Formaldehyde</th>
<th>Citric acid</th>
<th>TG</th>
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<td>0.5%</td>
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<td>-</td>
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</tr>
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<td>C2</td>
<td>15%</td>
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<td>0.2%</td>
<td>1%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
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<td>0.2%</td>
<td>2%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C4</td>
<td>15%</td>
<td>15%</td>
<td>0.2%</td>
<td>-</td>
<td>0.5%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C5</td>
<td>15%</td>
<td>15%</td>
<td>0.2%</td>
<td>-</td>
<td>1%</td>
<td>-</td>
<td>-</td>
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<td>15%</td>
<td>0.2%</td>
<td>-</td>
<td>2%</td>
<td>-</td>
<td>-</td>
</tr>
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<td>0.2%</td>
<td>-</td>
<td>-</td>
<td>0.5%</td>
<td>-</td>
</tr>
<tr>
<td>C8</td>
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<td>15%</td>
<td>0.2%</td>
<td>-</td>
<td>-</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
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<td>15%</td>
<td>0.2%</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
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<td>5 U/g</td>
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<tr>
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<td>0.2%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 U/g</td>
</tr>
</tbody>
</table>

Table 2.2: Composition of crosslinked (C) casein-based films. Each concentration of casein, glycerol, NaOH and crosslinkers, i.e. glutaraldehyde, formaldehyde, citric acid, transglutaminase (TG) is expressed in percentage by weight (wt%).

2.3 Film preparation

As mentioned before, different film compositions were studied. To make a film of 15% casein in 0.2% NaOH, four pellets of NaOH were dissolved in 200 ml of distilled water (DI-water). Then, 22.5 g of casein powder was dissolved in 15 ml of the 0.2% NaOH solution in a closed glass bottle with a magnetic stirrer bar, first, on a hot plate at 80°C for 2 hours and then in an ultrasonic bath at 60°C for 30 minutes. The ultrasonic bath allows one to remove air bubbles. Finally, the casein solution was filtered (190 micron pores) to remove all tiny and solid particles that may remain. In order to have a casein-based film mixed with 15% glycerol, 1.5 g glycerol was added to 8.5 g of casein solution. The casein-based solution was stirred on a hot plate at 60°C for 15 minutes. Lastly, the solution was refrigerated at 4°C for a few hours to ensure that most of the air bubbles were removed. The whole solution was poured in the center of two square containers of 8x8 cm until it covered the whole surface. Each film was left to air-dry at room temperature (21.5 ± 1°C) under the fumehood overnight.

This gentle process based on previous works is important to obtain a film of high quality [2,20].

de Decker Fanny
2.4 Crosslinking

In section 1.2.3, it was introduced that 4 crosslinking agents had been studied: glutaraldehyde, formaldehyde, citric acid and transglutaminase. These four components will be reused in order to compare their influence on the properties of casein-based films.

**Glutaraldehyde**

Similar to the crosslinked casein conduits in the work done by Wang et al. [23], a 25% glutaraldehyde solution was diluted in PBS to obtain 12.5% glutaraldehyde solution. This solution was mixed with the casein solution to have 0.5, 1 and 2% crosslinking. For 0.5% crosslinking, 2 g of glutaraldehyde solution was mixed with 50 g of casein solution. Then, the crosslinked solution was poured in the center of a square container until it covered the whole surface. Finally the solution was left to dry at room temperature (21.5 ± 1°C) under the fumehood overnight.

**Formaldehyde**

A 37.5% formaldehyde solution was mixed with casein solution to have 0.5, 1 and 2% crosslinking [33]. For 1% crosslinking, 1.35 g of formaldehyde solution was mixed with 50 g of casein solution. Then the crosslinked solution was poured in the center of a square container until it covered the whole surface. Finally the solution was left to dry at room temperature (21.5 ± 1°C) under the fumehood overnight.

**Citric acid**

For 2% citric acid crosslinking, 2 g of citric acid was diluted in 100 ml of DI-water. This citric acid solution was poured onto the dry casein film. The time duration depends on the citric acid concentration, the casein concentration and the casein film thickness. The citric acid solution needs to be removed when the casein film seems translucent to avoid the disbondement of the extremities of the film. The film was left to dry at room temperature (21.5 ± 1°C) under the fumehood overnight and then put in the oven at 150°C for 1 h 30 minutes.

**Transglutaminase**

There is approximately 7.5 g of casein for 65 g of casein solution for a film made up of 15% casein in 0.2% NaOH and 15% glycerol. Provided transglutaminase has an activity of 100 U/g, for a 10 U/g crosslinking, 0.75 g was needed. This quantity is diluted based on the rule 1 g in 10 ml of PBS. Then, the TG solution was mixed with 65 g of casein solution. Finally the solution was left to dry at room temperature (21.5 ± 1°C) under the fumehood overnight.
2.5 Gelatin/casein films

Another aspect of this project is aiming to develop 3D structures of casein. However, that was not easy with only casein solution due to its high content in water. When films were left to dry, water evaporated and left a very thin film. The result was not a stable 3D structure. To overcome this difficulty, casein solution was mixed with gelatin to add gelation properties and make possible 3D structures. Gelatin and casein have already been used together to produce crosslinked edible films [38].

A 15% casein solution in 0.2% NaOH solution, plus 15% glycerol was prepared similar to the method explained in section 2.3. In the mean time, a 15% gelatin solution in PBS was also prepared by dissolving 15 g gelatin in 100 ml PBS by stirring and heating at 60°C for 15 to 20 minutes to dissolve gelatin. Once gelatin was completely dissolved in PBS and the solution turned clear, the casein solution was prepared, the two solutions were mixed together with different weight proportions to form the hydrogel solution, which was then crosslinked by addition of TG solution. TG crosslinking was done in the same ways as explained in section 2.4 with a concentration of 10 U/g. Once the TG solution was added to the hydrogel solution, it was quickly mixed and poured in a square container. Finally, the solution was left to dry at room temperature (21.5 ± 1°C) under the fumehood overnight. Different weight proportions were prepared in order to analyze the influence of gelatin and casein on the mechanical, optical and physical characteristics. These are provided in Table 2.3.

<table>
<thead>
<tr>
<th>Casein solution</th>
<th>Gelatin solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 0%</td>
<td>100%</td>
</tr>
<tr>
<td>G2 33%</td>
<td>66%</td>
</tr>
<tr>
<td>G3 50%</td>
<td>50%</td>
</tr>
<tr>
<td>G4 66%</td>
<td>33%</td>
</tr>
<tr>
<td>G5 100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 2.3: Composition of gelatin/casein (G) based films. Each concentration of casein and gelatin solution is expressed in percentage by weight (wt%).

2.6 First observations

Similar characteristics were identified in films made up without plasticizer. All of these films were not flat and seemed hard, inducing lots of stresses in them, as shown in Figure 2.1. They seemed to vary in stiffness and thickness. NC1 film seemed very brittle, whereas NC5, NC11 and NC17 films seemed stiffer and thicker. Plasticizer, as its name implies, increases the plasticity of a material. Indeed, glycerol was used to increase the free volume of the polymer network and to render casein-based films more flexible. Consequently, this compound was needed to get films similar to plastic. Concerning NC6 and NC12 films, same results were observed as seen in Figure 2.2. Pure casein films tended to shrink during drying and became more brittle.
These films have not much interest in this work. Cell-culture substrates need to be flat and should not shatter.

Another feature that was directly observed was the softness of several films. NC3 and NC4 films looked like gelatin. NC4 remained in a liquid state after being left to air dry for 24 hours. This is represented in Figure 2.3 and can be explained by the high ratio of glycerol. These three films were not able to be peeled off the container. Consequently, these types of films were discarded for this project, since the bioprinting process cannot be done without peeling off films.

Specific visual characteristics could be observed for crosslinked casein-based films. As shown in Figures 2.4 and 2.6, glutaraldehyde and citric acid-crosslinked casein-based films had an orange-brown color. For citric acid films, the colour could be due to the baking of the films in order to crosslink them. For glutaraldehyde, the colour could be due to the chemical reaction of crosslinkage. In addition, citric acid-crosslinked casein-based films were rougher (holes, ridges)
than glutaraldehyde-crosslinked casein-based films. Formaldehyde and TG-crosslinked casein-based films were transparent, as seen in Figures 2.5 and 2.7. The latter were pretty similar to non-crosslinked casein-based films. At first sight, there were no differences between the different concentration levels of crosslinking agents except for glutaraldehyde. The higher the glutaraldehyde concentration level was, the rougher the film was.

![Figure 2.4: Glutaraldehyde-crosslinked casein-based films.](image)

Lastly, no significant differences were observed from the gelatin/casein films, as seen in Figure 2.8. All of them were clear and did not show any irregularities.

Eventually, a rule of thumb can be inferred from these basic observations, the higher the casein concentration level, the higher the film thickness and the tougher and more brittle the casein film. In addition, in a logical way, the higher the glycerol concentration level, the softer the film.
Figure 2.7: Transglutaminase-crosslinked casein-based films.

(a) C10 film.  
(b) C11 film.  
(c) C12 film.

Figure 2.8: Gelatin/casein films.

(a) G1 film.  
(b) G2 film.  
(c) G3 film.  
(d) G4 film.  
(e) G5 film.
Chapter 3

Mechanical properties

In cell-culture substrate stiffness affects various cellular aspects, such as morphology, migration, viability, growth, cytoskeletal structure and cell adherence [39, 40]. Stiffness can be defined as the rigidity of an object. The most common binding site for a mammalian cell is another similar cell or the extracellular matrix corresponding to a stiffness between 10 and 10000 Pa [9]. Most cells in multicellular organisms are usually attached to much softer materials than the glass and plastic surfaces used in vitro studies.

In this chapter, experimental setup for stiffness measurement is presented. Then, results are discussed.

3.1 Experimental setup

Mechanical properties can be measured in various ways. In this work, elastic property measurements, e.g stiffness, were performed by a Dynamic Mechanical Analysis (DMA) machine. It could have also been achieved by an Universal Testing Machine (UTM). The latter was not used due to the following reasons.

The UTM machine provides the stress-strain curve, as shown in Figure 3.1, in tensile mode for ductile materials. A ductile material is characterized by a plastic deformation before a fracture. This provides information about elastic behaviour of the material and its breaking strength. At first, a ductile material has an elastic behaviour meaning that has the ability to return to its original shape and size when forces are removed. This behaviour is described by a linear relation between the stress and strain and is known as Hooke’s law

\[ \sigma = E\varepsilon \]  

(3.1)

where \( \sigma \) is the stress, \( E \) the Young’s Modulus that characterizes the stiffness of the material and \( \varepsilon \) the strain. If more force is applied, material will reach the yield strength. Beyond this limit, material will behave plastically corresponding to an irreversible deformation before fracture.
The stiffness of each film could have been measured by an uniaxial tensile test using dumbbell-shaped specimen. Dumbbell-shape prevents failure in the grips and an accurate measurement by confining the deformation to the reduced section. However, casein films could not be cut directly into a dumbbell-shape with a scalpel because it induced different stresses in the film when the films became solid/dry directly in dumbbell shape. Hence, four different solutions were investigated before the right one was found.

First, a PDMS mould was cut into a dumbbell-shape with a scalpel and casein solutions were poured onto it afterwards. However, PDMS was not easy to cut with high accuracy and this method was discarded.

Another method was to pour PDMS on an aluminium mould that is patterned with a dumbbell-shape according to the plastic testing standard ISO527-1 [42]. PDMS was left to air dry at room temperature (21.5 ± 1°C) under the fume hood overnight and peeled off afterwards. However, it was impossible to correctly pour the casein solution onto the PDMS mould because it was too small. In the same manner, it was not easy to peel off the PDMS mould from the aluminium mould. In another way, casein solution was poured directly in the aluminium mould, but this did not work either as it was too sticky, tiny and thin.

A third method was investigated in which casein film was cut in dumbbell-shape with a cookie-cutter. The cookie-cutter was 3D printed in Acrylonitrile Butadiene Styrene (ABS), a thermoplastic polymer, as represented in Figure 3.2 (a). Size of the specimen was chosen according to [23]. However, the cookie-cutter was not sharp enough to cut the films easily (it required lots of pressure). Eventually, a PDMS mould was made in dumbbell shape via the cookie-cutter and casein solution was poured on it. PDMS base was mixed with the PDMS curing agent with a ratio of 10:1 for this. The solution was placed in vacuum for about 20 minutes for degassing. The PDMS was poured in the cookie-cutter on a flat surface and placed in vacuum for 1h 30 minutes. Then, the PDMS mould was left to crosslink on a hot plate for 2 hours at 80°C. After this the PDMS mould was removed from the cookie cutter and placed on a hot plate between two transparent sheets for 2 hours (see Figure 3.2 (b)). The casein solution was poured onto the PDMS mould and was dried at room temperature (21.5 ± 1°C) under the fume hood overnight, as shown in Figure 3.2 (c).

A solution was found to make dumbbell-shape specimen and ensure a constant section for a
homogeneous stress state. However, before placing the specimen between the grips, two dots would have to be drawn on the reduced section. These two dots are necessary to measure the change in gauge length during the test. A camera would film the displacement of these two dots. Knowing the strain and the applied stress, the stiffness would have been easily calculated. Therefore, the position of these two points is important and they need to be drawn in the right places. Finally, a lamp would have been used to light the workspace and have a good image quality, e.g. a sufficient contrast is necessary to follow the displacement of the markers at the surface of the specimen. This lamp would emit heat that would have affected the properties of casein-based films. This kind of measurement needs to be done several times to get reliable results, especially for these tiny samples. For all of these reasons, the DMA machine was preferred to the UTM machine.

Dynamic Mechanical Analysis consists of applying an oscillatory force to the specimen in a cyclic manner and reports change in stiffness and damping. The applied stress $\sigma$ elicits a corresponding deformation $\epsilon$ (strain) whose amplitude and phase shift $\delta$ can be determined, as
shown in Figure 3.3. From this, complex modulus $E^*$ is defined as

$$E^* = \frac{\sigma_A}{\epsilon_A} \tag{3.2}$$

It is composed of the storage modulus $E'$ (real part) and the loss modulus $E''$ (imaginary part)

$$E^* = E'(\omega) + iE''(\omega) = E^* \sin \delta + iE^* \cos \delta \tag{3.3}$$

where $\omega$ is the frequency of oscillation. Storage modulus is proportional to the energy stored during a loading cycle and can be interpreted as the stiffness of the material studied. On the other hand, loss modulus is proportional to the energy dissipated during a load cycle. Phase shift is the angle between the applied stress and the resultant strain. The damping is defined by the loss factor

$$\tan \delta = \frac{E''(\omega)}{E'(\omega)} \tag{3.4}$$

and compares how well a material will absorb or dissipate energy.

Dynamic mechanical analysis was conducted with a Dynamical Mechanical Analyzer Q800, TA Instruments equipped with a cryogenic system fed with liquid nitrogen. Liquid nitrogen was purchased from the Physics Department of the University of Canterbury, who have a $N_2$ liquid generator. Each film was cut into a rectangular specimen of 4x40 mm and conditioned for 48 hours at 23°C in 50% humidity (ambient condition). Only one sample per kind of film was tested due to the cost and the time it takes for each measurement. However, this kind of machine is known for its high accuracy. The testing procedure was based on ISO 6721-4 [44] and the DMA machine was used in tensile mode. The specimen was clamped at top and bottom and subjected to an underlying tensile stress to prevent it from buckling during dynamic load. Applied frequency was 1 Hz. The DMA machine provided the storage modulus, as well as the loss factor in function of temperature. Temperature scans from -100°C to 100°C were performed at a heating rate of 2°C/min.

### 3.2 Results and discussion

Dynamic Mechanical Analysis gave information about how viscous or elastic were the films via the storage modulus and the loss factor. Results for NC16 film are shown in Figure 3.4. These typical curves for amorphous polymer may be interpreted as follows: at low temperature, the molecules were so immobile that they were unable to resonate with the oscillatory loads and therefore remained stiff. This state is called the glass state. At elevated temperatures, the molecular segments became freely mobile and had no difficulty resonating with the load. This state is called the rubber state. A change from the glass state into the rubber state is called the glass transition. In the glass transition region, the storage modulus fell during heating. The material was able to storage less and less energy and its mechanical rigidity decreased.

Variations in storage modulus and glass transition temperature according the plasticizer...
were observed as reported in Tables 2.4 and 2.5 respectively. Glycerol broke the attachments that held the polymer chains together, facilitating the segmental mobility. Interactions of the protein chains in the films were reduced, and intermolecular spacing was increased, facilitating movement of the protein chains and leading to lower values of glass transition temperatures and storage modulus. These variations were previously observed for various polymers and protein materials [45–47].

All the films could not be assessed at the time of writing this thesis. Indeed, laboratory was closed for a few weeks due to an issue with the oxygen level in the room. According to the literature, influence of crosslinking would be characterized by an elevation of the transition glass temperature. This may be caused by molecular immobilization through covalent intermolecular crosslinking. Storage modulus would increase and films would become less viscous and less elastic [48]. Last tests are currently under investigation of the PhD student, Azadeh Hashemi.

All the available thermomechanical properties of the films are reported in Appendix B.
### Table 3.1: Storage modulus (MPa) at 37.5°C of non-crosslinked films made up of 15% casein as a function of plasticizer.

<table>
<thead>
<tr>
<th>Glycerol content (wt%)</th>
<th>$E'$ (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>171.70</td>
</tr>
<tr>
<td>15</td>
<td>16.22</td>
</tr>
<tr>
<td>25</td>
<td>2.98</td>
</tr>
</tbody>
</table>

### Table 3.2: Glass transition temperature (°C) of non-crosslinked films made up of 15% casein as a function of plasticizer.

<table>
<thead>
<tr>
<th>Glycerol content (wt%)</th>
<th>$T_g$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-49.82</td>
</tr>
<tr>
<td>15</td>
<td>-62.26</td>
</tr>
<tr>
<td>25</td>
<td>-77.48</td>
</tr>
</tbody>
</table>
Chapter 4

Optical properties

It is important to determine the optical properties of the material since the aim in cell-culture is to analyze and cultivate cells. Hence, a transparent material is preferred to visualize cells with standard microscope. In addition, casein were mentioned as autofluorescent according to Jain et al. [49]. Analysis of autofluorescence is necessary to ensure that fluorescence does not affect the cell analysis with dyes or labels. In short, optical properties are very interesting to study. In this thesis, transmittance and autofluorescence were investigated in particular.

In this chapter, experimental setup of transmittance and autofluorescence are first described. Then, results are discussed.

4.1 Experimental setup

4.1.1 Transmittance

As mentioned before, transmittance of the films need to be studied to determine the best film for use as cell-culture substrate.

Transmittance consists of measuring how much of a known incident light power ($P_0$) shone on a sample passes through it, as a function of the light wavelength. It can be expressed as a percentage by

$$T = \frac{P_T}{P_0} \times 100$$  (4.1)

where $P_T$ is the power of the light that passed through the sample and $T$ its transmittance. It is defined as the ratio of transmitted light to the incident light.

Spectrophotometry was used to investigate the properties of materials through their interaction with light, e.g. the intensity of the transmitted light. Different factors affect the values read on the detector. Light sources do not emit equal intensities of light on all wavelengths and detector response is a function of wavelength. Moreover mirrors, windows, gratings, and other optical components in the optical path of the spectrophotometer have efficiencies that vary with photon energy. Therefore, the influence of all these factors on the detector need to be known. These contributions to the spectrum not originated from the sample are called baseline. Besides
the contribution to the spectrum due to the baseline, another source of error on the determination of the absolute transmission is the non-zero response of the detector under dark conditions, namely the dark current.

Considering each sample has a substrate, the baseline measurement is done using a clean piece of substrate (blank sample). To measure the dark current of the detectors, the light path was blocked by using an opaque sheet. These measurements need to be done every time the spectrophotometer is used.

After measuring the spectrum from a sample \( T_0 \), the dark current of the detectors \( D \) was subtracted, then the raw spectrum obtained was divided by the baseline \( B \), also corrected for the dark current, to determine the contribution due solely to the sample, namely the absolute transmittance

\[
T = \frac{T_0 - D}{B - D}
\]  

Transmittance of each film was measured with a single beam UV spectrophotometer, the UV-Vis Cary 6000i, Agilent Technologies (see Figure 4.1). Each diffuse light transmittance measurement was made from 300 nm to 800 nm wavelength range to get data in the visible light range (400-700 nm). Data was recorded with a computer connected to the spectrophotometer and a graph of light transmittance percentage per nanometer was obtained by using the Scan software (version 4.20).

![Figure 4.1: Setup of the UV-Vis Cary 6000i spectrophotometer.](image)

4.1.2 Autofluorescence

Casein-based films were tested for their fluorescence properties to ensure that any autofluorescence of the material itself will not limit their utility while using fluorescent dyes for cell-culture and analysis.

Some molecules are capable of being excited to a higher energy state via absorption of light energy. The energy of the excited state cannot be sustained for long and decreases resulting in the emission of light energy [50]. More specifically, fluorescence is the result of a three-stage
process that occurs in certain molecules called fluorophores, as shown in Figure 4.2.

1. Excitation: A molecule has a relatively low energy and a stable configuration at the ground state $S_0$. When it absorbs a photon of sufficient energy $h\nu_{EX}$ supplied by an external source such as a laser, fluorophore moves to a higher energy state called an excited state electronic singlet state $S'_1$. Multiple excited states can be achieved depending on the wavelength and energy of the external light source. The molecule has an unstable configuration at excited state and it eventually adopts the lowest energy excited state (semi-stable).

2. Excited-state lifetime: Excited state electronic singlet state lasts for a very short time ranging from $10^{-15}$ to $10^{-9}$ seconds. Energy of $S'_1$ is partially dissipated (heating and other radiative processes), yielding a relaxed singlet excited state from which fluorescence emission originates.

3. Fluorescence emission: A photon of light is released returning the fluorophore to its ground state $S_0$. This photon has a lower energy $h\nu_{EM}$ (higher wavelength) than the exciting photon of light. The color of the light that is emitted is different from the color of the light that has been absorbed.

![Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence [50].](image)

Most fluorescence imaging is done using an epifluorescence microscope. 'Epi' comes from Greek and means 'same'. In this case, it means that both the excitation and the emission light travel through the same objective. The typical light path in an epifluorescence microscope is represented in Figure 4.3. Excitation light travels through a filter that narrows the wavelengths of the incoming light to only those used to excite the sample. This excitation light (in blue) is reflected by a dichroic mirror to the sample through the objective. The resultant emission light (in green) travels back through the objective and the dichroic mirror and then travels through the emission filter that transmits to the detector only the wavelengths of the emitted light from the sample and blocks all the light passed through the excitation filter. The detector is usually a Charge Coupled Device (CCD) camera.

\[ E = h\nu \]  
(4.3)

\[^{1}\text{The energy of an individual photon is given by}\]

where $h$ is Planck’s constant ($h = 6.626 \times 10^{-34}$ Js) and $\nu$ the frequency of the electromagnetic wave associated to the photon.
The fluorescence of each film was measured by the upright microscope Nikon Eclipse 80i, SDR Scientific (see Figure 4.4). Firstly, each film was analyzed by bright-field microscopy. That means white light is used and it relies on the differences in absorption of light due to differences in densities between various parts of the sample. Secondly, epifluorescence was investigated with an exposure time of 2 seconds and an 595 nanometers excitation light. All the measurements are done with a 10x eyepiece.

In fluorescence microscopy, image brightness is determined by the intensity of illumination, the quantum yield of the fluorophore, and the light-gathering power of the microscope. The greater the intensity of illumination and the higher the quantum yield, the greater the fluorescent signal and the brighter the image becomes until all of the fluorophores are saturated [51].

Images were analyzed by a Matlab script in order to know their intensity of brightness and consequently a percentage of autofluorescence of each film according to the exposure time. Each image had a size of 2048x2048 pixels and was converted in a matrix of pixels by the function \texttt{imread}. This matrix was averaged over its lines and columns by the function \texttt{mean2}. An intensity of 0% corresponds to a black photo and no fluorescence. An intensity of 100% corresponds to a white photo and 100% fluorescence meaning that fluorophores are saturated. The Matlab script is provided in Appendix A.

4.2 Results and discussion

4.2.1 Transmittance

Each casein-based film was made up of different compound concentration levels that influenced their appearance and consequently their transmittance. Results are intended to identified
the influence of casein, glycerol, buffer and crosslinking on the transmittance, as well as gelatin.

Transmittance as a function of wavelength provides two different pieces of information: opacity and color of the films. Indeed, from transmittance \( T \), absorbance \( A \) of the material can be deduced

\[
A = - \log_{10} \left( \frac{100}{T} \right)
\]  

(4.4)

Meaning that when transmittance is 100%, all the light passes through the material without any absorption, then absorbance is zero and then material is transparent. If all the light is absorbed, transmittance is zero and absorption is infinite (see Figure 4.5) and then material is opaque. In addition, a film is considered as clear (no color, white light) if all the wavelengths from the visible spectra are transmitted. The visible light ranges from 400 to 700 nanometers. Black corresponds to a total absence of light (no transmitted light). For instance, grass appears green because all of the colors in the visible range of the spectrum are absorbed into the leaves of the grass, except green. Green is reflected, therefore grass appears green. Visible light spectrum is represented in Figure 4.6. Each wavelength corresponds to a specific color.

![Figure 4.5: Relationship between absorbance and transmittance (%) [52].](image)

![Figure 4.6: Visible light spectrum [53].](image)
4.2.1.1 Influence of casein

Transmittance according to the wavelength from 300 nm to 800 nm of non-crosslinked casein-based films made up of 0, 5, 15 and 25% glycerol in 0.2% NaOH are presented in Figures 4.7, 4.8, 4.9 and 4.10 respectively.

![Graph](image)

Figure 4.7: Transmittance (%) according to the wavelength from 300 nm to 800 nm of films made up of 5, 10, 15 and 20% casein without glycerol.

Curves of transmittance depending on wavelength had similar patterns. Transmittance was zero at 300 nm meaning that the 300 nm light source was not transmitted by films. Then, transmittance increased significantly for a small range of wavelength and finally stabilized with a slight slope, which would have implied that films transmitted infrared rays (700 nm - 1 mm) and absorbed ultraviolet rays (10 nm - 300 nm). The higher the wavelength, the higher the transmittance.

For each graph, it could be seen that films made up of 5% casein had a higher transmittance than films made up of 10, 15 and 20% casein. Films made up of 10 and 15% casein had quite similar results even if films made up of 10% casein had a transmittance slightly higher. There was a significant difference of transmittance for the films made up of 20% casein. Their transmittance was lower, meaning films absorbed more light and were less transparent. Influence of casein on transmittance could be interpreted as higher the casein concentration level, lower the transmittance. This result could be directly observed on the films, as shown in Figure 4.11. NC2 film was more transparent than NC18 film.

Transmittance of NC4 film was not measured because, as mentioned before, NC4 film remained in liquid state after it was left to air dry which made the measurement impossible. Sample needed to be stand up vertically in the spectrophotometer so that the light beam can go through the film perpendicularly (see Figure 4.1). Moreover, an interference could be detected at 350 nm. This was due to the detector changeover of the spectrophotometer at this wavelength.
Figure 4.8: Transmittance (%) according to the wavelength from 300 nm to 800 nm of films made up of 5% glycerol and 5, 10, 15 and 20% casein.

Figure 4.9: Transmittance (%) according to the wavelength from 300 nm to 800 nm of films made up of 15% glycerol and 5, 10, 15 and 20% casein.

In short, transmittance decreased with the increase of casein concentration level. Films absorbed a larger amount of light and became more opaque, bordering on white. Casein powder was the compound that gives this whiteness (cf. milk). In addition, all of these films had no color due to the fact that all the wavelengths from the visible range were transmitted through the films.

4.2.1.2 Influence of glycerol

Transmittance according to the wavelength from 300 nm to 800 nm of non-crosslinked casein-based films made up of 5, 10, 15 and 20% casein in 0.2% NaOH are presented in Figures 4.12, 4.13, 4.14 and 4.15 respectively.
Figure 4.10: Transmittance (%) according to the wavelength from 300 nm to 800 nm of films made up of 25% glycerol and 10, 15 and 20% casein.

(a) NC2 film.  (b) NC18 film.

Figure 4.11: Influence of casein on transmittance of 5% glycerol casein-based films.

Figure 4.12: Transmittance (%) according to the wavelength from 300 nm to 800 nm of films made up of 5% casein and 0, 5 and 15% glycerol.

As mentioned before, curves have similar patterns. Transmittance increases significantly for small wavelengths and then stabilizes. For films made up of 5, 10 and 15% casein, it could be
seen that transmittance increases with glycerol concentration level. This statement could be directly deduced from the films, as shown in Figure 4.16.

![Graph showing transmittance (%)](image1)

Figure 4.13: Transmittance (%) according to the wavelength from 300nm to 800nm of films made up of 10% casein and 0, 5, 15 and 25% glycerol.

![Graph showing transmittance (%)](image2)

Figure 4.14: Transmittance (%) according to the wavelength from 300 nm to 800 nm of film made up of 15% casein and 0, 5, 15 and 25% glycerol.

An unexpected result was observed concerning films made up of 20% casein. Film with the higher concentration of glycerol had the lowest transmittance. This could be directly deduced observing the films as shown in Figure 4.17. NC20 film looked white compared to the other films. In short, the higher the glycerol concentration level, the higher the transmittance except for films made up of a minimum of 20% and 15% casein. This could be explain by a chemical reaction between casein and glycerol.

As mentioned before, all non-crosslinked films were colourless without any predominance of
transmittance (%) according to the wavelength from 300 nm to 800 nm of film made up of 20% casein and 0, 5, 15 and 25% glycerol.

Figure 4.16: Influence of glycerol on transmittance of 15% casein-based films.

(a) NC17 film.  (b) NC18 film.  (c) NC19 film.  (d) NC20 film.

Figure 4.17: Influence of glycerol on transmittance of 20% casein-based films.

a particular color given that all the wavelengths from the visible range were transmitted.

4.2.1.3 Influence of the buffer

Transmittance according to the wavelength from 300 nm to 800 nm of non-crosslinked casein-based films made up of 10 and 15% casein in 3% NaOH and 0.2%NaOH with 0 and 15% glycerol are presented in Figures 4.18 and 4.19, respectively.
Figure 4.18: Transmittance (%) according to the wavelength from 300 nm to 800 nm of films made up of 10% of casein and 0 and 15% of glycerol in NaOH and Na$_2$HPO$_4$ solution.

As observed for the other non-crosslinked casein-based films, transmittance decreased continuously as the wavelength shortened. Concerning films made up of 10% casein, a slight difference was observed between the two buffers. Transmittance was very similar from 450 nm to 800 nm. Between 300 nm and 450 nm, films made up of NaOH transmitted a larger amount of light. A more significant difference was observed for films made up of 15% casein. Films made up of Na$_2$HPO$_4$ had a lower transmittance than films made up of NaOH. In both cases, the higher the glycerol concentration level was, the higher the transmittance is as mentioned before. All the wavelengths from the visible range were transmitted and films had no colour. These slight differences could not be seen with the naked eye, as shown in Figure 4.20.

Figure 4.19: Transmittance (%) according to the wavelength (nm) of films made up of 15% of casein and 0 and 15% of glycerol in NaOH and Na$_2$HPO$_4$ solution.
4.2.1.4 Influence of crosslinking

Crosslinking is important to increase the degradation time of casein-based films. The influence of four crosslinking agents were investigated: glutaraldehyde, formaldehyde, citric acid and transglutaminase.

**Glutaraldehyde**

As shown in Figure 4.21, transmission in the visible range decreased significantly from non-crosslinked to glutaraldehyde-crosslinked films with a spectral shift corresponding to the sample color change from clear to orange-brown. Indeed, wavelengths from 400 to 550 nm corresponding to violet, blue and green colors were absorbed by the material (see Figure 4.6). In terms of crosslinker concentration level, a small difference could be distinguished. Films with higher crosslinker content exhibited lower transmittances. This could be explained by the fact that crosslinking agent made the material stronger. Films had a denser structure that affected the light transmission.

![Figure 4.21: Transmittance (%) according to the wavelength from 300 nm to 800 nm of glutaraldehyde-crosslinked films.](image-url)
Formaldehyde

Formaldehyde crosslinking decreased the transmittance of casein-based films, as seen in Figure 4.22. In terms of crosslinker agent concentration level, a slight difference could be distinguished. The higher the concentration level, the lower the transmittance. All the light sources from the visible light spectrum were transmitted. Consequently, films had no color.

![Figure 4.22: Transmittance (%) according to the wavelength from 300 nm to 800 nm of formaldehyde-crosslinked films.](image)

Citric acid

Transmittance of citric acid-crosslinked films was shifted compared to the non-crosslinked casein-based film, as depicted in Figure 4.23. There was no transmission between 300 nm and 420 nm. Citric acid and glutaraldehyde-crosslinked films had similar color. A lower amount of light was transmitted by citric acid-crosslinked films due to their roughness as noted in section 2.6.

Transglutaminase

Transglutaminase crosslinker agent decreased the transmittance of casein-based film, as shown in Figure 4.24. The concentration did not influence this reduction and all the light sources from the visible light spectrum were transmitted. Since all the wavelengths from the visible range were transmitted, transglutaminase-crosslinked films had no color.

In short, crosslinking decreased the transmittance of casein-based films in different ways according to the crosslinking agent. Crosslinking exhibited a protective effect against visible light. As mentioned before, crosslinking increased film stability and density structure of the films. Light diffusion into a denser structure was decreased, lowering optical transmittance.
Figure 4.23: Transmittance (%) according to the wavelength from 300 nm to 800 nm of citric acid-crosslinked films.

Figure 4.24: Transmittance (%) according to the wavelength from 300 nm to 800 nm of TG-crosslinked films.

4.2.1.5 Influence of gelatin

Transmittance according to the wavelength from 300 nm to 800 nm of films made up of a mixture of gelatin and casein in the proportion 1:0, 3:1, 1:1, 1:3 and 0:1 respectively are presented in Figure 4.25. From these results, the following could be deducted. Concerning gelatin/casein films, the gelatin concentration level increased light transmission. Moreover, pure gelatin and pure casein films transmitted a larger amount of light. Pure gelatin film had the highest transmittance. Another remark is the gelatin based film transmitted light at 300 nm. The low transmittance of gelatin/casein films under visible light indicated a poor compatibility between gelatin and casein, which also indicated that the gelatin/casein composite films were less transparent.
To conclude, except glutaraldehyde and citric acid casein-based films, all the films were clear and could have advantages in cell-culture substrate applications. Cells could be visualized with standard transmission light microscopes. Moreover, this kind of material could be used in ophthalmic applications e.g. contact lenses.

4.2.2 Autofluorescence

Devices were tested for their fluorescence properties to ensure that any autofluorescence of the material itself will not limit its utility while using fluorescent dyes for cellular analysis. Fluorescence images were analyzed via a Matlab script to highlight and compare their intensity of brightness and get a percentage of autofluorescence in function of the exposure time (see section 4.1.2). As a reminder, an intensity of 100% corresponds to a white photo meaning that the fluorophores are saturated. Inversely, an intensity of 0% corresponds to a black photo meaning no fluorescence.

Autofluorescence intensities of non-crosslinked casein-based films for an exposure time of 2 seconds are shown in Figures 4.26 (a) and (b). Non-crosslinked films made up of different concentration of casein in NaOH solution mixed with different concentration of glycerol had a low autofluorescence, between 7% and 17% of intensity. It could be noticed that the casein and glycerol concentration levels did not influence the autofluorescence of the films and, intensity of fluorescence was very similar for different concentrations of casein and glycerol. Concerning films made up with another buffer, there were no huge difference. Autofluorescence intensities varied between 8% and 11%. The buffer did not noticeably influence the autofluorescence of casein-based films.

A comparison of the autofluorescence intensity of casein-based films crosslinked with glutaraldehyde, formaldehyde, citric acid and TG and a non-crosslinked film is shown in Figure 4.27 (a) for 2 seconds of exposure time. A weak intensity of fluorescence, below non-crosslinked
Figure 4.26: Autofluorescence of non-crosslinked casein-based films - Exposure time of 2 s, eyepiece 10x.

casein-based films (about 12%), was noticed for formaldehyde and TG-crosslinked casein-based film.

Figure 4.27: Autofluorescence of crosslinked casein-based films - Exposure time of 2 s.

A saturated intensity of brightness was observed for glutaraldehyde- and citric acid-crosslinked casein-based films. Fluorophore were saturated with an exposure time of 2 seconds (100% of intensity). In order to compare the fluorescence of these films, the exposure time was reduced. Autofluorescence intensities of glutaraldehyde- and citric acid-crosslinked films are presented in Figures 4.28 and 4.29 respectively. Glutaraldehyde-crosslinked films had a higher autofluorescence than citric acid-crosslinked casein-based films [54]. Exposure time was reduced to 50 ms and 150 ms for glutaraldehyde-crosslinked films and citric acid-crosslinked films respectively. Concerning concentration level of crosslinker, a higher concentration level induced a high autofluorescence and inversely as observed directly on the photos.

Autofluorescence intensities of G1, G2, G3 and G4 films were 9.01, 8.01, 6.86 and 6.27%
respectively. Gelatin/casein films were slightly autofluorescent. The ability of the gelatin to fluoresce at different wavelengths has been determined by Yova et al. [55]. However, these intensities were too low to interfere with cellular analysis using fluorescent dyes.

To conclude, casein-based films were slightly autofluorescent. The parameter that greatly affected the autofluorescence was the type of crosslinker. Glutaraldehyde and citric acid induced a large autofluorescence of the film. Consequently, these films will not be used as cell-culture substrates for cellular analysis using fluorescent dyes or labels. On the other hand, casein, gelatin and glycerol concentration levels and buffers did not influence the film autofluorescence as much. Autofluorescence of the material was shown to be minimal and within the range of typical background, ensuring utility with analyses using fluorescent dyes and labels would not be affected.

### 4.2.3 Bright-field microscopy images

In the same time as fluorescence, each film was analyzed by bright-field microscopy. Figures 4.30 shows bright-field microscopy images of non-crosslinked casein-based films with a 10x eyepiece. It could be seen that films were different and this difference varied in function of casein and glycerol concentration level as well as buffer. In a future work, AFM could be used to investigate the roughness of the surface of films according to these parameters.

Bright-field microscopy images of crosslinked casein-based films show that films also looked
Figure 4.30: Optical micrographs of non-crosslinked casein-based films, eyepiece 10x.

different in function of crosslinkers and their concentration. Images are represented in Figure 4.31. Same statement was noticed for gelatin/casein films as shown in Figure 4.32.

Figure 4.31: Optical micrographs of crosslinked casein-based films, eyepiece 10x.
Figure 4.32: Optical micrographs of gelatin/casein films, eyepiece 10x.
Chapter 5

Physical properties

Physical properties are major in the characterization of a material. In this work, contact angle, water uptake ratio and degradation time were studied. First, experimental setup is described. Then, results are discussed.

5.1 Experimental setup

5.1.1 Contact angle

In this thesis, the biomaterial should enhance growth and implantation of the surrounding tissue for a better performance in biocompatibility. Consequently, cell adhesion should be especially good. Adhesion properties of micro-organisms onto a biomaterial can be evaluated by using contact angle measurements. In a previous works, optimal cell adherence onto polymers was observed for contact angles between 45 and 75 degrees [7, 56–58].

Consider a liquid drop resting on a flat, horizontal solid surface. The contact angle is defined as the angle formed by the intersection of the liquid-solid interface and the liquid-vapor interface. Therefore, contact angle is geometrically acquired by applying a tangent line from the contact point along the liquid-vapor interface in the droplet profile. The interface where solid, liquid, and vapor co-exist is referred to as the “threephase contact line”, as shown in Figure 5.1.

As first described by T. Young in 1805, the contact angle of a liquid drop on an ideal solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions

\[ \sigma_{lv} \cos \theta = \sigma_{sv} - \sigma_{sl} \]  

(5.1)

where \( \theta \) is the contact angle and \( \sigma_{lv}, \sigma_{sv} \) and \( \sigma_{sl} \) represent the liquid-vapor, solid-vapor, and solid-liquid interfacial tensions, respectively.

Contact angle indicates the degree of wetting. Small contact angles (\(< 90^\circ\)) correspond to high wettability (the solid material is considered as hydrophilic), while large contact angles (\(> 90^\circ\)) correspond to low wettability (the solid material is considered as hydrophobic). It is
represented in Figure 5.1.

![Figure 5.1: Contact angle [36].](image)

In this thesis, water contact angle measurements were performed by a telescope-goniometer CAM 2008, KSV Instruments. It consists in static mode using sessile drop method by placing a 1 μl drop of DI-water on the sample surface which was mounted on an illuminated stage. The measurement was achieved by simply aligning the tangent of the sessile drop profile at the contact point with the surface and reading the protractor through the eyepiece via the CAM 2008 software. Each casein film was cut into a strip of 25x75 mm and stuck onto a glass microscope slide with double-faced tape to be as flat as possible. A minimum of three measurements on the same sample was done. The setup is illustrated in Figure 5.2.

![Figure 5.2: Contact angle measurement setup.](image)

5.1.2 Degradation time and water uptake ratio

Considering casein is a water soluble protein, it is helpful to analyze the behaviour of the films when they are immersed in DI-water, or even media. It is also important to analyze the degradation time of crosslinked casein-based films according to the crosslinker type and concentration. Depending on the type of medical implant, the degradation time is different.

Water uptake ratio is expressed as a percentage increase of weight after immersion in DI-water for a predetermined time. The dry films were cut into circles with a diameter of 2.54 cm and weighed. Then, they were immersed in DI-water at room temperature (21.5 ± 1°C). The swollen films were then removed from water at predetermined times, blotted dry and weighed. The percentage of weight was calculated using the following equation

$$\Delta W = \frac{W_t - W_0}{W_0} \times 100 \quad (5.2)$$
where $W_0$ and $W_t$ indicate the weight of the film before and after immersion in water at a predetermined time $t$. To be able to weigh films easily they were placed onto a wire mesh of 50 mm width (ZKW-MON 50/10, Farnell) and nails were used to take the mesh out of the liquid (see Figure 5.3). Water absorption was followed in every experiment by weighing until equilibrium had been attained (about 24 hours).

At the same time, the degradation time of each sample was observed, as well as the expansion of the diameter of each sample. Indeed, swelling can occur in $x$ and $y$ direction. Consequently, it is useful to know the expansion of the films that influences the features. The expansion is given in percentage and is calculated by measuring the diameter of the films, at each time and using the equation below

$$\Delta E = \frac{D_t - D_0}{D_0} \times 100$$

(5.3)

where $D_t$ and $D_0$ are the diameter of the film at any given time $t$ and the diameter of the film before immersion into liquid, respectively.

In the same way, these three characteristics (water uptake ratio, degradation time and expansion of diameter) were investigated with films immersed in cell-culture media at 37.5°C. Cell-culture media was prepared by adding 10% Fetal Bovine Serum (FBS), 1% fungizone and 1% Penicillin/Streptomycin (P/S) solution to Dulbecco’s Modified Eagle Medium (DMEM).

5.2 Results and discussion

5.2.1 Contact angle

In cell-culture, most of the cells have a substrate attachment dependence. Contact angle is one of the parameters which can characterize the cell adhesion onto a material [7, 56–58]. A hydrophilic material would be preferred for supporting cell growth.

Contact angle was measured on dry films in order to know the hydrophilicity or hydrophobicity of casein-based films. It was impossible to measure the contact angle of some films meaning as NC3, NC4 and NC10, due to their softness. They were not able to be peeled off and put on a glass slide. And it was impossible to measure the contact angle of film in the container because...
of edges of the container. Camera could detect the container walls and not the surface of the films. Finally, NC12 was too curvey and not flat to measure the contact angle.

Contact angle of non-crosslinked casein-based films are shown in Figure 5.4. Additional casein-based films with 10 and 20% of glycerol concentration level mixed with 5, 10, 15 and 20% of casein were made in order to try to understand the influence of the parameters on the contact angle (test 2). However, there was no direct relationship to be seen. Measurements were made 3 times on the same film and repeated on 3 different films. Contact angles were very different between the different measurements. This might be explained by the fact that casein absorbs water and thus moisture. Consequently, the time between the film making and the measurement influenced the contact angle as well as impurities, the room temperature and humidity. Results for contact angle of non-crosslinked films were not relevant. A solution could have been, making all the films in a controlled environment and measure the contact angle after a predetermined time. In addition, film making and contact angle measurements were done in different laboratories. Films were moved from place to place in different environments. They should have been put in a conditioning box.

As seen in Figure 5.5, contact angles of crosslinked casein-based films did not vary much with respect to the concentration of each crosslinking agents. In addition, crosslinking increased the contact angle and consequently the hydrophobicity of the films, which should not be a good thing. Indeed, hydrophilic biomaterial promotes good cell spreading, enhances cell attachment as mentioned in section 5.1.1.

In addition, as discussed in section 1.2.3, cells adhere onto TG-crosslinked casein-based films. TG has the highest contact angle, about 94°, corresponding to a hydrophobic material. That means there was another parameter (chemical reaction, leaching, ...) that influenced the cell adhesion onto the film. Water droplet on non crosslinked and TG-crosslinked casein-based film are represented in Figure 5.6. It can be seen droplet spread more on non-crosslinked casein-based film.

Figure 5.4: Contact angle (in degree) of non-crosslinked casein-based films.

As seen in Figure 5.5, contact angles of crosslinked casein-based films did not vary much with respect to the concentration of each crosslinking agents. In addition, crosslinking increased the contact angle and consequently the hydrophobicity of the films, which should not be a good thing. Indeed, hydrophilic biomaterial promotes good cell spreading, enhances cell attachment as mentioned in section 5.1.1.

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Figure 5.5: Contact angle (in degree) of crosslinked casein-based films. Left: contact angle of different concentration of crosslinked casein-based films. Concentrations are 0.5, 1 and 2% for glutaraldehyde, formaldehyde and citric acid crosslinker and 1 U/g, 5 U/g and 10 U/g for transglutaminase crosslinker. Right: average of 3 contact angle of different concentration of crosslinker for each crosslinker.

Figure 5.6: Water droplet on a non-crosslinked casein-based film (left) and a TG-crosslinked casein-based film (right).

Contact angles of gelatin/casein films are listed in Table 2.6.

<table>
<thead>
<tr>
<th>Film</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>83.92</td>
</tr>
<tr>
<td>G2</td>
<td>130.80</td>
</tr>
<tr>
<td>G3</td>
<td>109.33</td>
</tr>
<tr>
<td>G4</td>
<td>105.71</td>
</tr>
<tr>
<td>G5</td>
<td>96.38</td>
</tr>
</tbody>
</table>

Table 5.1: Contact angle (°) of gelatin/casein films.

Given the potential applications in this work, films will be used in a liquid environment. As already mentioned, casein has the property to absorb water that influences directly the film hydrophilicity when film is immersed. Once films have absorbed water, they can be considered
hydrophilic and contact angle has not a high importance.

To conclude, contact angle properties could not be used to characterize cell adhesion onto casein-based films. However, about crosslinked casein film, it could be deduced that there was something else that would be the reason of favorable cell adhesion.

### 5.2.2 Degradation time and water uptake ratio

Casein-based films are biodegradable and casein absorbs water. In a logical way, degradation time and water uptake ratio of casein-based films were analysed.

The degradation of casein-based films were investigated in DI-water at room temperature (21.5 ± 1°C) and in cell-culture media at 37.5°C. As expected, the main difference was due to the crosslinking as will be explained below. As a reminder, crosslinking increased the stability of the polymer that means it retains its original structure and function within a sufficient period of time. Consequently, the degradation time of non-crosslinked and crosslinked casein-based film were evaluated separately.

#### 5.2.2.1 In DI-water

**Non-crosslinked films**

First of all, the degradation time and consequently the water uptake ratio of NC3, NC4 and NC10 films were not analyzed. As mentioned before, these films were too soft. They could not be peeled off and consequently, be immersed in DI-water.

<table>
<thead>
<tr>
<th>Casein (%)</th>
<th>Glycerol(%)</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>70 min</td>
<td>40 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>100 min</td>
<td>60 min</td>
<td>50 min</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>120 min</td>
<td>80 min</td>
<td>60 min</td>
<td>30 min</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>120 min</td>
<td>80 min</td>
<td>70 min</td>
<td>50 min</td>
</tr>
</tbody>
</table>

Table 5.2: Degradation time (in minute) of non-crosslinked casein-based films in DI-water at room temperature (21.5 ± 1°C).

Non-crosslinked casein-based films were dissolved in less than 110 minutes as described in Table 2.7. Influence of casein and glycerol were deduced. The higher casein concentration level, the longer degradation time. The higher glycerol concentration level, the shorter degradation time. For instance, 20% casein-based film made up of 0, 5, 15 and 25% glycerol were dissolved in 120, 80, 70 and 50 minutes respectively. Glycerol has three hydroxyl groups that are responsible
for its solubility in water and its hygroscopic nature [59]. These could lead to an increase in water diffusion in the films and consequently, an increase in their solubility [?]. Casein could be considered as the solidifying compound.

![Graph 1](image1)

**Figure 5.7**: Water uptake ratio (%) of 5% casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) between t = 0 min and t = 24 h.

![Graph 2](image2)

**Figure 5.8**: Water uptake ratio (%) of 10% casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) between t = 0 min and t = 24 h.

Water uptake ratio of 5, 10, 15 and 20% casein-based films are shown in Figures 5.7, 5.8, 5.9 and 5.10 respectively. For each film, the weight of the film increased to a certain point and then started to decrease. Each film absorbed water during the first 10 minutes and then started to degrade and lost its integrity. For example, casein-based film of 20% casein and 5% (NC18) and 25% (NC20) glycerol in NaOH solution swelled to about 504% and 94% respectively. Then, they dissolved rapidly. Evolution over time of NC18 and NC20 films immersed in DI-water are represented in Figures 5.11 and 5.12 respectively. For a same concentration of casein, the higher
As shown in Figure 5.9 and Figure 5.10, the water uptake ratio (%) of casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) decreases with increasing glycerol concentration. This is evident from the expansion of the films. The film with 0% glycerol (NC17) swelled to 186%, whereas the films with higher glycerol concentrations swelled less. It can be concluded that the lower the casein concentration, the lower the water uptake ratio. Indeed, the compound of the film known for its properties of water absorption was the casein powder. In a logical way, if there is a larger amount of casein powder (higher concentration), the film will absorb more water and consequently the water uptake ratio will be higher and the film will swell more.

In short, the degradation time and the water uptake ratio of non-crosslinked casein-based films were affected by glycerol and casein concentration levels. As already mentioned, casein is water soluble and dissolves by absorbing water. Glycerol is not water soluble but it has a hydrophilic nature, which explains the observed behavior.
Figure 5.11: Swelling and degradation over time of NC18 film immersed in DI-water at room temperature (21.5 ± 1°C).

Figure 5.12: Swelling and degradation over time of NC20 film immersed in DI-water at room temperature (21.5 ± 1°C).

Figure 5.13: Swelling and degradation over time of NC2 film immersed in DI-water at room temperature (21.5 ± 1°C).

drophilic plasticizer. Glycerol increases hydroxyl groups available to make hydrogen bond with water and enhance the solubility of the casein-based films in DI-water. In addition, glycerol is hygroscopic [59] and did not prevent swelling of the films. Both parameters increased the degradation time and water uptake ratio of the films but casein had a major impact.

**Crosslinked films**

The degradation time and the water absorption of a film is strongly influenced by the crosslinked microstructure of the polymers and the presence of hydrophilic groups.
Table 5.3: Degradation time (in minute) of crosslinked casein-based films in DI-water at room temperature (21.5 ± 1°C).

Overall, the degradation time of crosslinked casein-based films was longer than non-crosslinked casein-based films as shown in Table 2.8. Casein-based films crosslinked with 1 U/g and 5 U/g transglutaminase dissolved in DI-water in 70 and 160 minutes respectively. This could be explained by the fact that the concentration of crosslinker was not sufficient to crosslink the film and increased their stability. All the other films could be considered as crosslinked. Formaldehyde and 10 U/g TG casein-based films were dissolved in nearly two months. Citric acid and glutaraldehyde lost their integrity after more than two months. In addition, a slight difference in degradation time could be observed between 0.5% and 1-2% of crosslinker. Higher the crosslinker concentration level, higher the degradation time.

Given their long degradation time, the effect of the water uptake ratio was easily observed over a 24-hour period. Water uptake ratio was influenced in a different way according to the crosslinking reagent as will be explained below.

**Glutaraldehyde**

Water uptake ratio over a 24-hour period for glutaraldehyde-crosslinked casein-based films is represented in Figure 5.14. Films absorbed water fast during the first hour of immersion and then the water uptake ratio stabilized. This water absorption was characterized by the swelling of the films as shown in Figures 5.15 and 5.16. Higher concentration of crosslinker induced a lower water absorption. For instance, 0.5% and 2% glutaraldehyde-crosslinked casein-based films swelled to about 179% and 384% respectively. The water absorption decreased with the increase in the amount of crosslinking agent in the film. This was a commonly observed phenomenon and attributable to the fact that with the increase in the amount of crosslinker, the number of crosslinks between the chains increases, thus reducing the free space available for incoming water molecules [45, 60]. This resulted in decrease in the water absorption. One more reason was that with the increase in the extent of crosslinking, the number of crosslink points along the casein chains increased, thus rendering the chains stiffness. As a result, the relaxation of casein chains was suppressed, thus resulting in decrease in the water uptake.
Figure 5.14: Water uptake ratio (%) of glutaraldehyde-crosslinked casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) between $t = 0$ min and $t = 24$ h.

Figure 5.15: Swelling and degradation over time of C1 film immersed in DI-water at room temperature (21.5 ± 1°C).

Figure 5.16: Swelling and degradation over time of C3 film immersed in DI-water at room temperature (21.5 ± 1°C).

Formaldehyde

Water uptake ratio over a 24-hour period for formaldehyde-crosslinked casein-based films is represented in Figure 5.17. The amount of water absorption for formaldehyde films was quite small and did not exceed 50%. This result can be interpreted by Figures 5.18 and 5.19. Films did not swelling a lot. They absorbed rapidly water and then stabilized. In addition, a slight difference could be observed between glutaraldehyde crosslinker concentration. Higher the crosslinker concentration, lower the swelling of the films.
Figure 5.17: Water uptake ratio (%) of formaldehyde-crosslinked casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) between t = 0 min and t = 24 h.

Figure 5.18: Swelling and degradation over time of C4 film immersed in DI-water at room temperature (21.5 ± 1°C).

Figure 5.19: Swelling and degradation over time of C6 film immersed in DI-water at room temperature (21.5 ± 1°C).

**Citric acid**

Water uptake ratio over a 24-hour period for citric acid-crosslinked casein-based films is represented in Figure 5.20. Films swelled about to 80% that means they did not expand that much as shown in Figure 5.21 and 5.22. In addition, it could be observed a decrease of the absorbed water over time meaning a slight deterioration of the films and finally a stabilization. This could explain the degradation of patterns when films were immersed in liquid. First few nano/micro layers of the films would have started to degrade (see section 1.2.3.3). The crosslinker concentration influenced slightly the water uptake ratio. Moreover, it could be observed films became opaque after 10 minutes of immersion in DI-water.
Figure 5.20: Water uptake ratio (%) of citric acid-crosslinked casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) between t = 0 min and t = 24 h.

Figure 5.21: Swelling and degradation over time of C7 film immersed in DI-water at room temperature (21.5 ± 1°C).

Figure 5.22: Swelling and degradation over time of C9 film immersed in DI-water at room temperature (21.5 ± 1°C).

Transglutaminase

Water uptake ratio over a 24-hour period for transglutaminase-crosslinked casein-based films is represented in Figure 5.23. As mentioned before, 1 U/g and 5 U/g of transglutaminase were not sufficient to crosslink casein-based films. C10 and C11 films swelled to about 208% and 304% respectively and then dissolved rapidly. C12 film swelled to about 1300%. These expansions over time can be seen in Figures 5.24 and 5.25. They looked like gelatin and their thickness increased a lot.

In brief, crosslinking influenced water absorption of casein-based films in different ways according to the crosslinking reagent. Films could swell a lot such as TG-crosslinked films, or not absorb water very much, such as formaldehyde and citric acid-crosslinked casein-based films.
Figure 5.23: Water uptake ratio (%) of TG-crosslinked casein-based films immersed in DI-water at room temperature (21.5 ± 1°C) between $t = 0$ min and $t = 24$ h.

Figure 5.24: Swelling and degradation over time of C10 film immersed in DI-water at room temperature (21.5 ± 1°C).

Figure 5.25: Evolution over time of C12 film immersed in DI-water at room temperature (21.5 ± 1°C).

Swelling of the films would induce a deterioration of patterns (see Appendix C).

**Influence of gelatin**

Gelatin was used to make 3D structure of casein. The degradation time of gelatin/casein films are reported in Table 2.9. An increase of casein concentration induced a slight decrease of degradation time of the gelatin/casein films. The reason of this result is still not fully understood.

Water uptake ratio over a 24-hour period for TG-crosslinked gelatin/casein films is represented in Figure 5.26. It could be seen that casein increased the water absorption of the films. Indeed, as already seen, casein had the ability to take in large quantities of water and gelatin did
Table 5.4: Degradation time (in minute) of gelatin/casein films in DI-water at room temperature (21.5 ± 1°C).

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
</tr>
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<tbody>
<tr>
<td>days</td>
<td>65</td>
<td>57</td>
<td>50</td>
<td>38</td>
<td>58</td>
</tr>
</tbody>
</table>

Figure 5.26: Water uptake ratio (%) of gelatin/casein films immersed in DI-water at room temperature (21.5 ± 1°C) between t=0min and t=24h.

not prevent this water absorption. G2 and G4 films swelled about to 37% and 149% respectively meaning in a larger expansion of G4 film as shown in Figures 5.27 and 5.28. In a logical way, G5 film (100% casein - 0% gelatin) had a high water uptake ratio. In addition, G1 film (0% casein - 100% gelatin) had a water uptake ratio almost as high as G3 film. It swelled about to 95%. In brief, it could be seen that if casein is added to gelatin, water uptake ratio can be increased or decreased according to the casein concentration level.

Figure 5.27: Swelling and degradation over time of G2 film immersed in DI-water at room temperature (21.5 ± 1°C).

5.2.2.2 In media

Degradation time of casein-based films was also studied in media at 37.5 degrees in order to mimic the body temperature. Similar to the degradation time of films in DI-water (see section 5.2.2.1), crosslinked casein-based film had a much longer degradation time than non-crosslinked casein-based films.
Figure 5.28: Evolution over time of G4 film immersed in DI-water at room temperature (21.5 ± 1°C).

As presented in Table 2.10, degradation time of non-crosslinked casein-based films was longer than in DI-water. For instance, films made up of 20% casein and 5% glycerol was dissolved in 80 and 210 minutes in DI-water and media respectively. This could be explained by the fact that media was composed of a lower amount of water. Moreover, it was easy to see each films swelled less. Unfortunately, quality of pictures of the films immersed in media was bad. Consequently, picture were not presented.

<table>
<thead>
<tr>
<th>Glycerol(%)</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>80 min</td>
<td>40 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>150 min</td>
<td>70 min</td>
<td>50 min</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>160 min</td>
<td>150 min</td>
<td>60 min</td>
<td>30 min</td>
</tr>
<tr>
<td>20</td>
<td>210 min</td>
<td>210 min</td>
<td>150 min</td>
<td>90 min</td>
</tr>
</tbody>
</table>

Table 5.5: Degradation time (in minute) of non-crosslinked casein-based films in media at 37.5°C.

About crosslinked films, similar results were obtained. Degradation time of these films was higher as reported in Table 2.11.

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>0.5% (or 1 U/g)</th>
<th>1% (or 5 U/g)</th>
<th>2% (or 10 U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citric acid</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>120 min</td>
<td>200 min</td>
<td>&gt; 2 months&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not dissolved at the time of writing this thesis yet. Films are under the supervision of Azadeh Hashemi now.

Table 5.6: Degradation time (in minute) of crosslinked casein-based films in media at 37.5°C.

In addition, water uptake ratio of crosslinked casein-based films immersed in media was lower compared to films immersed in DI-water. Figures 5.29, 5.30, 5.31 and 5.32 shows water

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de Decker Fanny    72
absorption of glutaraldehyde, formaldehyde, citric acid and TG-crosslinked films over 24 hours, respectively. The larger swelling difference could be noticed in TG-crosslinked casein-based films. For 10 U/g, films swelled almost 13 times (1271%) from its original weight in DI-water and only 2 times (228%) in media. Glutaraldehyde-crosslinked casein-based films swelled in media half as much as in DI-water. In media, formaldehyde-crosslinked films absorbed water and then stabilized between 11% and 19%. On the other hand, citric acid-crosslinked films absorbed water, then a decrease of the absorbed water could be observed and finally their water uptake ratio stabilized (25 - 39%).

Figure 5.29: Water uptake ratio (%) of glutaraldehyde-crosslinked casein-based films immersed in media at 37.5°C between t = 0 min and t = 24 h.

Figure 5.30: Water uptake ratio (%) of formaldehyde-crosslinked casein-based films immersed in media at 37.5°C between t = 0 min and t = 24 h.

Degradation time of gelatin/casein films did not be assessed because media was infected. These tests are currently under investigation.
Figure 5.31: Water uptake ratio (%) of citric acid-crosslinked casein-based films immersed in media at 37.5°C between t = 0 min and t = 24 h.

Figure 5.32: Water uptake ratio (%) of TG-crosslinked casein-based films immersed in media at 37.5°C between t = 0 min and t = 24 h.

Unexpected results were observed about the water uptake ratio of gelatin/casein films. Water uptake ratio of gelatin/casein films immersed in media is shown in Figure 5.33. Films absorbed more water in media than in DI-water. The reverse was observed for all the other films. It can also be noted that higher the gelatin concentration is, higher the water uptake ratio is. Due to the fact that an infection was observed after several days, these results might be wrong. These experiments need to be carried out again in order to get reliable results.

5.2.3 Expansion of diameter

Even after crosslinking, the films were still biodegradable, which means films absorbed water once they were immersed in liquid. This can also lead to swelling of the films and thus a change
in the size of the films and any features on the surface (see Appendix C). To investigate this, how the films crosslinked with each crosslinker behaved when immersed into either media or DI-water were investigated. Figure 5.34 shows the expansion of the diameter of the crosslinked casein devices as a function of crosslinking reagents, immersion liquid and time.

The maximum expansion of film diameter after 24 hours in water or media, ranged from 12% of the original diameter, for formaldehyde or citric acid-crosslinked films in water or media, to almost 100% for TG-crosslinked film in water. Other studies, investigating water absorption of
casein films, based on the change in the weight of the films, have shown casein films crosslinked via some of aldehyde crosslinkers (i.e. glyceraldehyde and glutaraldehyde) could absorb water up to four or five times their original weight once immersed in water [20], which consequently influenced the size of the films as observed in this work.

![Graph showing expansion of diameter of gelatin/casein film](image)

Figure 5.35: Expansion of diameter of gelatin/casein film in proportion 1:1 in water and media over 24 hours.

The expansion of diameter of gelatin/casein film in proportion 1:1 in function of liquid and time is shown in Figure 5.35. Gelatin/casein film diameter did expand around 4% and 7% in media and DI-water, respectively.

To conclude, the diameter of citric acid and formaldehyde-crosslinked films and gelatin/casein film in proportion 1:1 did expand slightly in DI-water and media and might not influence the patterns. Expansion diameter of glutaraldehyde and TG-crosslinked films was greatly reduced in media compared to DI-water that could be an advantage.
Chapter 6

Conclusion and future work

Biodegradable casein-based films were made of different concentration levels and were characterized in order to understand the influence of each compound of the casein film and optimize the films for use as cell-culture substrates and medical implants. As previously seen, a challenge was encountered as cells did not properly adhere to the films. This explains the reason of such a characterization work. Besides, it is also required to know all the characteristics of a new material.

In order to fulfill this objective, different tests were performed. These tests were chosen according to the potential applications.

Stiffness of a material affects various cellular aspects, such as morphology, migration, viability, growth, cytoskeletal structure and cell adherence. The most common binding site for a mammalian cell is another similar cell or the extracellular matrix corresponding to a stiffness between 10 and 10000 Pa. That is why mechanical properties are very important parameters to be studied.

Moreover, a transparent material is preferred to visualize cells with standard microscope. Consequently, transmittance of the material was analyzed. Actually, it needs to be high for all wavelengths in visible range.

Analysis of autofluorescence was also necessary to ensure that fluorescence does not affect the cell analysis with dyes or labels.

Then, adhesion properties of micro-organisms onto a biomaterial were evaluated by using contact angle measurements. Previous studies have shown that optimal cell adherence onto polymers was observed for contact angles between 45 and 75 degrees.

Lastly, degradation time of the films was examined. It needs to be sufficient and swelling of the immersed films needs to be low in order to have no deformation of the 3D cells imprints.

In theory, an ideal cell culture substrate would be a transparent, no fluorescent and biocompatible material. The main required characteristics for a medical implant would be biocompatibility and suitable mechanical properties depending on its function.

Unfortunately, no film responded to all of these characteristics. In this work, it has been seen that only TG-crosslinked casein-based film was cell-friendly despite its high contact angle. It had a high transmittance in visible light that enables to visualize cells with standard microscopes. Another advantage is the fact that patterns on TG-crosslinked casein-based film did not expand
much in media (about 30%). This might provide bioimprints similar to the cultured cells and enhance the biocompatibility of the material.

Note that biodegradability of the films could be controlled by variation of the crosslinker concentration. Higher the crosslinker concentration is, higher the degradation time is.

For instance, casein-based film crosslinked with 10 U/g of TG dissolved in almost two months. That could be an appropriate degradation time for cell-culture substrate. Actually, this latter film had almost all the necessary characteristics for cell-culture substrate and can be considered as the best film for use as cell-culture substrate.

In addition to the properties used in this thesis to characterize biodegradable casein-based films, other tests could be performed. Indeed, in order to determine which film is the best for potential use as medical implants, it could be of interest to study the ultimate tensile strength (in other words, the maximum stress a material can withstand without tearing) of casein-based films. Depending on their function, medical implants require different mechanical properties to avoid failure and to perform well.

The mechanical properties of all the films were measured in air at standard atmospheric conditions only. It would be also of interest to know the mechanical behavior of casein-based films in cell-culture media due to the potential medical implant and cell-culture substrate applications in order to mimic the body environment and get results closer to reality.

This work also provides a panel of results that could be used for other studies such as the transition glass of this polymer.

Considering the development of biodegradable casein-based microdevices, cells cultured on different substrates (one made of biodegradable TG-crosslinked casein, which will expand and the other, made of polystyrene, which will retain its original size) are currently in the process of investigating. Cells behaviour on these patterned films needs to be analyzed in order to visualize the differentiation of the cells and to figure out if the 30% of swelling influence the cell differentiation.

As a final step, those experiments should be translated to the clinics. It is important to remark the ethic aspects and the great amount of regulations that have to be met in order to launch the biomaterial to the market.
Bibliography


[47] International standard iso 6721-4 - determination of dynamic mechanical properties - part 4: Tensile vibration.


Appendix A

Matlab script for autofluorescence

% Analysis of Fluorescence Images
% 2017 Louise Orcheston–Findlay/Fanny de Decker
% Casein film autofluorescence analysis
% clc % clear all input and output from the command windows
% close all % close the current figures
% clear all % clear matlab workspace

dirlist = dir('*.tif'); % create a structured dirlist of all .tif images in the folder
imageSize = 2048; % size of .tif images

for x = 1:length(dirlist)
a = char(dirlist(x).name); % extract image name from structure
imageArray(x).name = a; % create structure imageArray with name = image name
end

for x = 1:length(dirlist)
crop = imread(imageArray(x).name); % create pixel matrix of image
pixels = imcrop(crop); % crop image to focus on the region of interest (center)
pixels = ((double(pixels) + 1)/65536)*100; % convert pixels into intensity (in percent)
imageArray(x).imageMean = mean2(pixels); % mean of each intensity matrix
end

% sorting of each results according to glycerol concentration
j=1;
for i=1:4
  glycerol0(i)=imageArray(j).imageMean;
glycerol5(i)=imageArray(j+4).imageMean;
glycerol15(i)=imageArray(j+8).imageMean;
glycerol25(i)=imageArray(j+12).imageMean;
j=j+1;
end
% creation of concentration vector
concentrationcasein = [5 10 15 20];

% create figure – fluorescence intensity of each film in function of casein concentration
figure
hold on
plot(concentrationcasein, glycerol0, 'o')
plot(concentrationcasein, glycerol15, 'o')
plot(concentrationcasein, glycerol25, 'o')
legend('0% glycerol','5% glycerol','15% glycerol','25% glycerol')
xlabel('Concentration of casein (%)')
ylabel('Intensity of autofluorescence (%)')
axis([0 25 3 17])
hold off

% sorting of each results according to casein concentration
k=1;
for i=1:4
    casein5(i)=imageArray(k).imageMean;
    casein10(i)=imageArray(k+4).imageMean;
    casein15(i)=imageArray(k+8).imageMean;
    casein20(i)=imageArray(k+12).imageMean;
k=k+1;
end

% creation of concentration vectors
concentrationglycerol = [0 5 15 25];

% create figure – fluorescence intensity of each film in function of glycerol concentration
figure
hold on
plot(concentrationglycerol, casein5, 'o')
plot(concentrationglycerol, casein10, 'o')
plot(concentrationglycerol, casein15, 'o')
plot(concentrationglycerol, casein20, 'o')
legend('5% casein','10% casein','15% casein','20% casein')
xlabel('Concentration of glycerol (%)')
ylabel('Intensity of autofluorescence (%)')
axis([-5 30 5 17])
hold off
Appendix B

Results from the Dynamic Mechanical Analyzer

The thermomechanical properties (i.e. storage modulus and loss factor) of the casein-based films were determined by Dynamic Mechanical Analysis (see Chapter 3). Each film was cut into a rectangular specimen of 4x40 mm and conditioned for 48 hours at 23°C in 50% humidity (ambient condition). The specimen was clamped at top and bottom and subjected to an underlying tensile stress to prevent it from buckling during dynamic load. Applied frequency was 1 Hertz. The DMA machine provided the storage modulus, as well as the loss factor in function of temperature. Temperature scans from -100°C to 100°C were performed at a heating rate of 2°C/min. All the results are reported below.
Figure B.1: Thermomechanical properties of NC1 film.

Figure B.2: Thermomechanical properties of NC2 film.
Figure B.3: Thermomechanical properties of NC8 film.

Figure B.4: Thermomechanical properties of NC9 film.
Figure B.5: Thermomechanical properties of NC13 film.

Figure B.6: Thermomechanical properties of NC14 film.
Figure B.7: Thermomechanical properties of NC15 film.
Appendix C

Features expansion

Surface topography is increasingly recognized as an important parameter which can influence cell phenotype [12]. A method used to print cells on the material surface has been developed. It aimed to promote adhesion of these cells on the surface, as well as improve biocompatibility and growth. In other words, it allows to mimic the natural cell environment.

Regular features e.g. crosses, were transferred on different casein-based films. The process consists of different steps as fabrication of intermediate moulds with geometric patterns by photolithography and soft lithography (photoresist, mask, liquid-casting). It was adapted from [18] as explained in section 1.2.2.

As mentioned in section 5.2.2, casein-based films swelled when they are immersed in water. It is important to analyse the expansion of the features on the surface of the films to understand how the features could influence the cell adhesion. Cells adhere to TG-crosslinked casein-based films as mentioned in section 1.2.3. Moreover, gelatin/casein films were developed to get some 3D structures. The features expansion of TG-crosslinked films and gelatin/casein films were investigated. Films were patterned and then immersed in water and media. A Olympus BX30 microscope was used to get optical micrographs of the feature at predetermined time.

The measured change in the size of the cross feature on TG-crosslinked casein-based films immersed in media and water is plotted in Figure C.1. Interestingly, films immersed in water expanded almost three times more than films immersed in media. When immersed in DI-water the pattern has disappeared at 24 hours, while it is still present in media. However, the reason for this behaviour is still unclear and is currently being studied. Optical micrographs showing the change in the size of a cross-shaped test feature replicated on gelatin/casein films in the proportion 1:0 and 1:1 are presented in Figure C.2 (a) and (b), respectively. It can be seen the pattern still there at 24 hours and did not change in size. This result is directly related to the low diameter expansion of casein/gelation film (see section 5.2.3).

While this amount of swelling results in a change in the size of the features, it is yet to be seen how this change would influence the cultured cells. To understand this better, cells cultured on casein substrates with pattern sizes adjusted during fabrication, as well as on different substrates,
Figure C.1: Effect of water absorption on surface patterns. (a) Optical micrographs showing the change in the size of a cross-shaped test feature replicated on TG crosslinked casein film, immersed in media and DI-water within 24 hours. (b) Measured change of dimensions of the example feature on TG cross-linked casein film after immersion in water and media within 24 hours.

one made of biodegradable casein, which will expand and the other, made of polystyrene, which will retain its original size are currently in the process of investigating.
Figure C.2: Optical micrographs showing the change in the size of a cross-shaped test feature replicated on casein/gelatin crosslinked casein film, immersed in media and DI-water within 24 hours.