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MASTER'S THESIS

Materials Science and Engineering

3D-PRINTED ALGINATE-PEPTIDE SCAFFOLDS FOR BONE TISSUE ENGINEERING

Memòria i Annexos

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Resumen

Uno de los principales retos de la ingeniería tisular es generar andamios que proporcionen propiedades biológicas óptimas para imitar la matriz extracelular. El objetivo de este proyecto es desarrollar biotintas de alginato modificado con péptidos RGD para imprimir en 3D biomateriales para la ingeniería óseotisular. Con este fin, en primer lugar, se ha realizado y mejorado un protocolo de funcionalización del alginato y se han elaborado hidrogeles modelo en 2D. Estos hidrogeles han sido caracterizados a nivel fisicoquímico mediante XPS y microscopia confocal de fluorescencia, y a nivel biológico con ensayos celulares in vitro. Posteriormente se crearon filamentos 3D y se volvieron a realizar caracterizaciones biológicas. Siendo la cantidad de péptido pequeña, su presencia con la caracterización física y química fue difícil de probar. Sin embargo, los resultados biológicos de los hidrogeles modelo en 2D exitosos pusieron de manifiesto la presencia de péptido y su repartición homogénea en el hidrogel. Se destaca que los hidrogeles funcionalizados con RGD mostraron una mejora en la adhesión y proliferación celular en relación a los controles. Así, una mejor bioactividad fue encontrada gracias al añadimiento de la secuencia de péptido RGD. Sin embargo, los resultados sobre los filamentos se demostraron poco fiables, así es necesario llevar a cabo otras técnicas de caracterización como el radiomarcaje, la radioactividad o ensayos biológicos con células humanas para corroborar los resultados de este proyecto.

Abstract

A main challenge of Tissue engineering is to create sustainable scaffolds that provide optimal biological properties to mimic the extracellular matrix (ECM). The aim of this project is to develop RGD modified alginate bioinks for creating 3D-printed scaffolds for bone tissue engineering. To achieve this purpose, a functionalisation protocol has been performed and improved and 2D biofilms have been elaborated. Then, physicochemical characterisation methods such as XPS and fluorescence confocal microscopy were carried out on films together with in vitro cell-based assays. After that, 3D filaments were created and biological characterisations once again carried out. The amount of peptide being little, its presence with physical and chemical characterisation was hard to prove. However, successful biological results on biofilms spotlighted the presence of peptide and its homogeneous repartition in the hydrogel. It has also been detected that RGD functionalised films showed a great cell adhesion and spreading comparing to non-functionalised ones. Therefore, a better bioactivity was found after addition of RGD peptide sequences. However, filaments results being unreliable, further characterisation technics such as radiolabelling, radioactivity or biological assays with human cells have to be carried out to corroborate the results of this project.

Glossary

ECM – Extracellular Matrix

HA – Hydroxiapatite

NCP – Non Collageneous Proteins

TE – Tissue Engineering

RGD – Arginine-Glycine-Aspartic

HA – Hydroxiapatite

PVA – Polyvinyl Alcohol

PEG – Polyethylene Glycol

hBMSC – Human Bone Marrow Stromal Cells

EDC – 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

NHS – N-hydroxysuccinimide

PU – Polyurethane

PBS – Phosphate Buffered Saline

FTIR – Fourier Transform InfraRed spectroscopy

DMEM – Dulbecco/Vogt modified Eagle's minimal essential medium

FBS – Fetal Bovine Serum

PFA – Perfluoroalkoxy Polymer

RCF – Relative Centrifugal Force

XPS – X-Ray Photoelectron spectroscopy

LCSM – Laser Confocal Scanning Microscopy

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1. Introduction

Bone is defined as a living tissue that is part of the vertebrate skeleton. It provides structure for the body and enables mobility but is also important because it provides red and white blood cells and store minerals. The extracellular matrix (ECM) of bones is made of 65% of inorganic materials in weight such as hydroxyapatite (HA) and around 25% of organic phase which is mainly composed of 90% of collagen and 10 % of non-collagenous proteins (NCP) such as fibronectin, osteopontin and osteocalcin. These proteins play a significant role in cellular interactions and therefore in regeneration. The last 10% of the ECM is basically water. [1]

As bone is a smart tissue, the organic part of the ECM is able to regenerate and heal slowly the bone. However, critical-size defects caused by fractures, traffic accidents or age-related conditions, for example, are unable to heal and require external help. Tissue engineering (TE) aims at repairing and regenerating impaired organs or damaged tissues. It has the goal of replacing and improving biological tissues with a combination of biochemical and physical factors, the use of cells and employing biomaterials. This field includes several disciplines such as material and mechanical engineering, but also biomedical science. [2]

Originally, to achieve the regenerative goal of TE, autografts and allografts were developed and consisted respectively, of implanting a part of a patient's bone such as ribs or hips or using bone from a deceased donor or cadavers. However, both present several limitations, including painful procedures for autografts, and infection and rejection risks for allografts. Then, Xenografts which are grafts derived from other species were used but also have rejection risks. This is why TE requires the use of a natural or biodegradable synthetic structure called a scaffold which presents lower risks of infection/rejection and does not require a second surgery. [1] [2]

2. Requirements

2.1. Scaffolds

A scaffold is an artificial extracellular matrix that will be built in vitro in order to be implanted in vivo in the human body and thus provide the mechanical support and bioactivity that will enhance the generation of tissue while degrading itself. It represents a biocompatible and biodegradable structure that will be implanted in the patient's body to achieve the main goal of TE: regenerative therapy. This scaffold has to take the shape and the structure of the human tissue it will replace and thereby act as the ECM. Other requirements of a scaffold, as it should mimic the ECM, would be the porosity of the material in order to provide vascularization to the tissue and promote the transport of active biomolecules such as cells and nutrients. Also, mechanical properties are important in order for the scaffold to be maintained on the tissue defect and ease the implantation without degrading itself before. Finally, bioactivity of the material is required with the aim of allowing cell attachment and proliferation. Indeed, the biomaterial has to be cell-adhesive so it can have an impact on cells. Last but not least, a scaffold should also provide the biochemical and biological signals required for the regeneration. However, the release has to be well controlled, for example, controlling the crosslinking of the encapsulation material. [2] [3]

2 types of scaffolds exist. Firstly, they can be made of synthetic materials, either polymers or ceramics, which can create very stable bond with the human tissue but have some drawbacks. Indeed, they have, for example, lower compatibility and ceramics are brittle and hard to shape. The second type are natural scaffolds made either of proteins or polysaccharides. These biomaterials are in general much more biocompatible and bioactive, except from alginate. In this project, this polysaccharide will be used but combined with Arginine-Glycine-Aspartic acid sequence peptide (RGD) in order to obtain a bioactive property. [4] [5]

Scaffolds in TE can be used for a very large number of medical applications. For example, hydroxyapatite (HA) is the main inorganic material used for bone engineering thanks to its high biocompatibility and its osteoconductive properties. Tricalcium phosphate is also used for its absence of rejection and its ability to provide calcium and phosphorus to new tissues. These 2 materials are used in cranial and spinal surgery and for repairing fractures. However, as it was said previously said, their insufficient biocompatibility and sometimes biodegradability are limiting their applications, especially because they are inorganic materials and so cannot interact with cells. That is why they need to be combined with synthetic or natural polymers to be more efficient. A concrete example would be PLA-HA scaffolds studied by S. Tanodekaew and co. in 2013 that highlight the excellent properties of HA in term of tissue bonding and using PLA polymer in order to enhance cell adhesion and provide better biocompatibility and biodegradation. [6] Finally, ceramics are not always required for scaffolds to provide bone regeneration, indeed lots of studies about biocompatible polymers linked with biomolecules such as chitosan or gelatin containing proteins were carried out and are explained in the alginate part of this report. This will be the case of our study as it will require the use of a hydrogel of alginate that will be the polymer and RGD-peptide the biomolecule. [7] [8]

2.2. Hydrogels

Hydrogels are defined as 3D cross-linked networks composed of hydrophilic polymers that are strongly and chemically linked (covalent or ionic bonds). These polymers are capable of absorbing a high amount of water. They can be either natural, including proteins and polysaccharides. Or they can be synthesized artificially such as Polyvinyl Alcohol (PVA) or Polyethylene Glycol (PEG). However, the second type of hydrogel require complex processes of synthesis and a modification of the matrix in order to be biocompatible even though they have a higher absorption content. [9]

They have a wide range of applications such as agriculture, food additives, artificial snow but what we will focus on during this project is their use in the biomedical field such as their pharmaceuticals, drug delivery systems but especially TE applications. For this last one, they are widely used because of their similarities to the ECM that are principally constituted of collagen fibres and proteoglycan filaments, they represent the structural support and the environment where cells attach and proliferate but also give the mechanical properties such as elasticity and rigidity to the tissue. Concerning hydrogels, they can mimic the ECM thanks to their interwoven structure because they can be derived from collagen or fibrin that are naturally present in the human body or coming from natural sources such as alginate (brown algae) or chitosan (shellfish). [8] [9]

Naturals hydrogels, the type that will be used in this project, have great ability of cellular attachment and proliferation, indeed they offer abundant space and a perfect environment for cells to grow. They give mechanical constitution to the tissue and permit cells attachment and proliferation as the cells can either attach to the surface of the hydrogel or be suspended in the 3D network. Moreover, the space present in this network allow cells to pass but also nutriments and proteins required for regenerative therapy. They are also biodegradable and biocompatible and thanks to their mimetic properties, are perfect scaffolding materials. Moreover, their ease to be transformed into scaffolds comes from their ability to be chemically or physically crosslinked and form most of the time covalent bond to give a better mechanical behaviour to the material. Thus, it facilitates its implantation in the human body and help keeping its posture in order to be maintained in the located zone where regeneration is needed. [10] [11]

Concerning applications of hydrogels for TE, they are numerous and continue to expand nowadays. For example, Mredha et al. delivered a dual network hydrogel (chemically and physically crosslinked) with collagen fibril and polyetherimide for cartilage [12]. Another example is Co ions and Bone Morphogenetic proteins core shell fibres hydrogels scaffolds for rat calvarium defects delivered by Perez et al. in 2015. But more examples will be given in the next parts. [13]

2.3. Alginate

Alginate is a natural polysaccharide composed of M (mannuronic) and G (guluronic) blocks as it can be seen in *figure 2.1*. It can have several forms: alternating MG residues or consecutive M or G residues. It is one of the most abundant marine biopolymers and comes from brown algae. Its extraction consists first of a bath in sulfuric acid in order to liberate the alginic acid and the second step of a carbonatation that enable the alginic acid to be solubilised in sodium alginate and so the algae residues can be taken off. Finally, sulfuric acid is added once again to insolubilize the sodium alginate and allow its filtration. [14]

This polymer is widely used in hydrogels thanks to its high capacity to absorb water and its easy crosslinking with help of cations such as Ca^{2+} as it can be seen on *figure 2.2*. Other interesting aspects of alginate are its biocompatibility properties, low price and ease to be sterilized and stored. But most of all, it is an excellent candidate for TE applications because of its flexibility. Indeed, it can be transformed and crosslinked with simple chemical and physical reactions. Thus, it can have various structures and properties depending on the other reactants. Also, alginate enables a great control of porosity and size of pores thanks to its viscosity range and its easy crosslinking, so the cell can attach and proliferate easily. Thus, it has an excellent ability to be 3D-printed. Its crosslinking with calcium chloride renders alginate with good mechanical strength so it can be used as a drug or biomolecules delivery material. Moreover, this last advantage is very important so the scaffold can be manipulated and handle the surgery without breaking. However, even though alginate possesses varying biocompatibilities in vitro, it is not a bioactive material. In other words, it does not interact with cells, it acts as a bioinert substrate for TE which means it contains no cell receptors and no possibilities to send signals to cells. This is the reason why, it needs to be combined with bioactive substances such as gelatine, chitosan or molecules such as proteins or amino acids sequences with affinity for cell receptors before being implanted in the human body to acquire the properties required. [3] [14] [15]

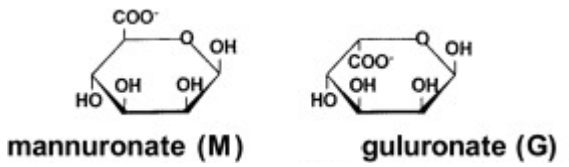

Figure 2.1: chemical structure of M and G alginate blocks.

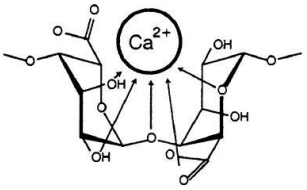

Figure 2.2: Alginate crosslink with Ca^{2+}

The applications of alginate are numerous. It is used in the food industry as softening agent for breads or thickener for sauces but also in clinical field for dental impression moulds and in the pharmaceutical field for health food and weight loss supplements. But in this case, the most interesting applications are made in TE such as cell encapsulation and drug or proteins delivery materials as it was said before thanks to their biocompatibility of course but also because the release of substances can be very well controlled depending on its degradation rate and so the way it is crosslinked, the pH, etc. Indeed, it can take the shape of microspheres, microcapsules, sponges, foams or fibres in addition to hydrogels. [15] [16]

Concerning the past uses of this polymer, Alginate has been studied a lot in the biomedical field and especially for TE. Indeed, in 1997 alginate “sponges” for tissue regeneration were studied by Ms. Shapiro

and M. Cohen. They mentioned the biocompatibility of alginate and its great structural properties that enable the sponge to maintain the shape during bone regeneration. However, they also found after a cell culture that fibroblasts cell attached to the sponge but kept a spherical shape and did not spread which is due to the non-bioactive property of alginate discussed earlier [17]. For that reason, alginate needs to be functionalized with bioactive molecules that interact with cell receptors. Indeed, these bioactive molecules will enable the material to interact with cells when attached to alginate as it can be seen in *figure 2.3.* [15] [16] [18]

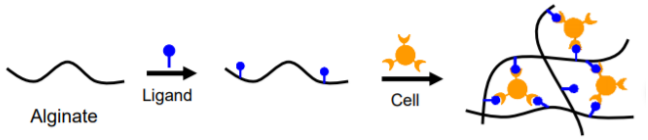

Figure 2.3: Cell-crosslinked network formation after functionalising alginate. [19]

Therefore, assays were carried out on alginate functionalised with other bioactive molecules such as gelatin. Luo Y. et al. investigated this alginate-gelatin mixture in 2015 and the results of the cell culture are shown in *figure 2.4.* [20]

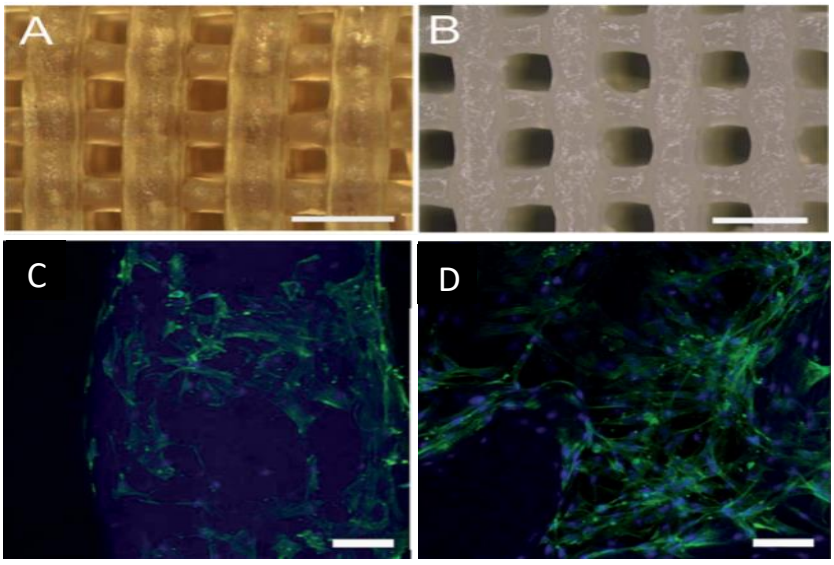

Figure 2.4: Scaffold structure of pure alginate (A) and gelatin/alginate (B)
Confocal pictures of hBMSC (Human Bone Marrow Stromal Cells) seeded at day 1 on alginate (C) and gelatin alginate (D) scaffold. [20]

As it can be seen in the figure, much more fluorescent human Bone Marrow Stromal Cells (hBMSC) were attached and proliferated in the scaffold containing gelatin (D) than pure alginate (C). It is therefore clear that gelatin promotes attachment and proliferation of cells and thus helps the scaffold to be bioactive.

Other studies were also carried out in 2005 on alginate-chitosan scaffolds and results were quite similar; proving a better attachment and proliferation of cells on an alginate scaffold when it is functionalized with bioactive molecules [21]. Another practical example would be the bio-ink alginate/alginate sulphate developed by Park J. et al. in 2018, whose aim was to deliver BMP proteins for bone tissue engineering as a growth factor delivery and showed that alginate sulphate helped the release of proteins in order to promote regeneration and osteogenesis. [22]

2.4. RGD-Peptide

Peptides are chains composed of several amino acids linked together by amide bonds.
In this project, the focus will be done on the arginylglycylaspartic acid (RGD) that can be seen in *figure 2.5.* It is one of the most common peptides in terms of supporting cell adhesion and proliferation thanks to its combination of 3 amino acids: Arginine, Glycine and Aspartate. [23]

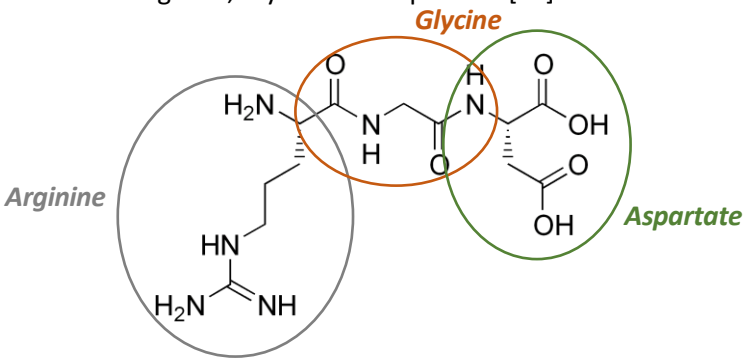

Figure 2.5: RGD peptide molecule.

The use of this motif is quite recent as solid-phase peptide synthesis were achieved only in 1959 and the identification of the RGD sequence discovered in 1984. It is in part for this reason that in the beginning of TE, only proteins such as fibronectin or collagen were coated on biocompatible polymers in order to give bioactivity to the scaffold. But there were some drawbacks in the use of proteins: they need to be isolated as they can create undesirable immune responses. Also, their application is limited in time because they need to be refreshed continuously because of their self-degradation. Over time, these problems were solved thanks to the use of peptides and their motifs attracting cell receptors, that possess better general stability in the ECM. [23]

Concerning previous studies about RGD scaffolds, a study was performed using RGD-functionalized polyurethane scaffolds by A. Tahlawi et al., showing great results in term of osteogenic differentiation.[24] Another study was also carried out in 2012 by W-B. Tsai et al. conjugating this time RGD with chitosan. They proved lots of benefits that bring the fact of adding RGD in a scaffold such as promoting cell adhesion and proliferation but also osteogenic differentiation and mineralization and some of the results are shown in *figure 2.6*. [25] [26]

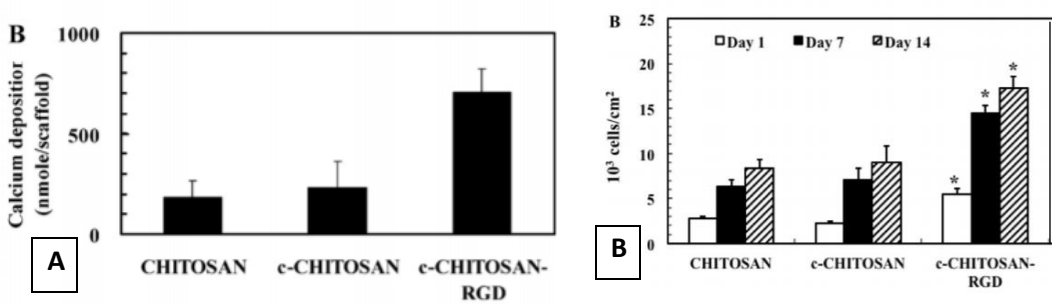

Figure 2.6: Calcium deposition by MSCs on chitosan unmodified, azido-modified and conjugated with RGD after 14 days (A) and density of cells attached on the same type of samples (B). [26]

For example, the results showed above reveal the excellent mineralization properties of RGD, as it allowed a better calcium deposition (*figure 2.6.A*). It also promoted a better cell attachment and proliferation (*figure 2.6.B*) as showed by a higher density of cells on a chitosan-RGD surface than on pure chitosan at day 1 as well as at day 14. But these cellular concepts will be explained more clearly in the bioactivity part of the report.

2.4.1. Bio-Functionalisation of alginate with RGD

As previously said, alginate will be bio-functionalised with the RGD motif peptide with the goal of mimicking the bones ECM, giving to the scaffold the bioactivity that was not initially present. An interesting aspect of the RGD molecule is its ease to react with hydroxyl, amino and carboxylic groups. In our case for example, it will be useful as alginate has carboxylic groups. Indeed, the reaction occurring between alginate and peptide with the help of *EDC* activator is shown in *figure 2.7* (**reaction I**) and leads to the 2 reactants linked by an amide bond which represents our bio-functionalised product. However, a secondary reaction appears and is basically the hydrolysis (**reaction II**) of the primary product *o-Acylisourea Intermediate* into alginate because of the water present in the solution. The fact is that this secondary reaction is way more stable than the one desired. A third reaction (**reaction III**) will then be artificially added thanks to the NHS chemical in order to produce an *amine reactive ester* that will then react with the nitrogen of the peptide. The EDC/NHS chemical is therefore compulsory to achieve a good performance of the functionalisation. [25] [27]

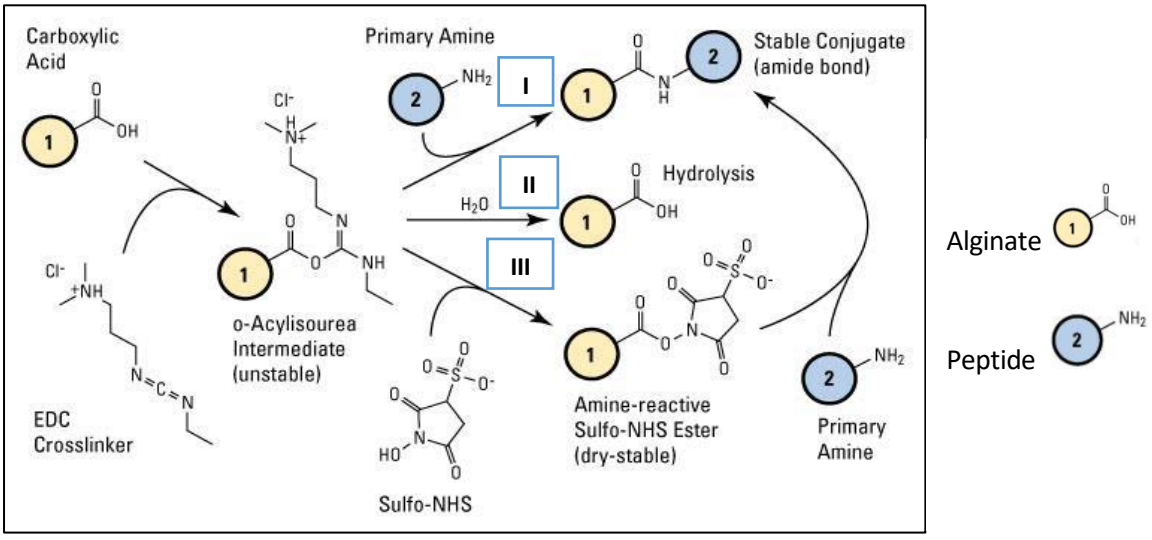

Figure 2.7: Functionalisation reaction of alginate with a peptide linked with an amide group. [28]

This reaction was already studied and optimized in 1999 by Mooney et al. thanks to several experiments with various parameters (pH, concentration, activation etc.). Culture cells were also carried out on alginate and RGD modified alginate surfaces and the results are shown in *figure 2.8*. [29]

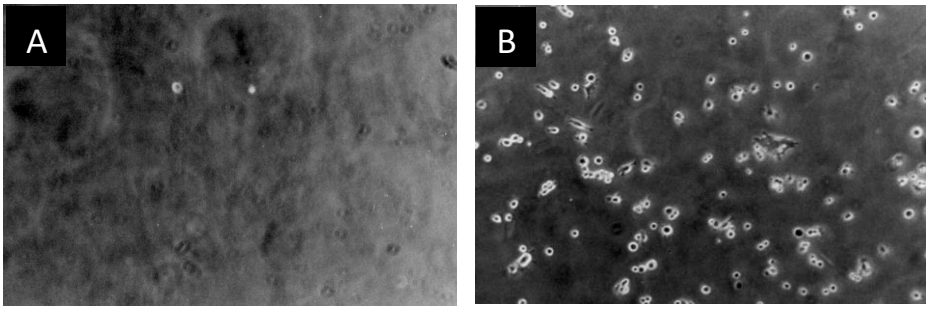

Figure 2.8: Myoblast cells adherent to control alginate surface (A) and RGD modified alginate surface (B) at 4h post-seeding. [29]

Almost no myoblast adhesion was observed on the control surface whereas the alginate modified with peptide seems much more attractive for cells because they look well attached and began even spreading only 4h after seeding. The absence of cells confirms the fact that alginate is not bioactive as it can be seen in the *figure 2.8.a* whereas the surface showed in *figure 2.8.b* react with cells. These results proved then the presence of RGD peptide on the surface and so highlight the fact that the crosslinking protocol worked. [29]

2.4.2. Bioactivity

Thanks to RGD motif bioactivity, which means its ability to interact with living tissues, it has been shown in 2003 by Huang H and his colleagues that the RGD sequence plays a significant role in cell attachment and proliferation. In addition, these results confirm the ones found by Mooney and co that are discussed just above. Moreover, when it is incorporated within a scaffold, this peptide sequence may also bring osteogenic differentiation and mineralization. The first means an ability to self-renew for cells and the capacity to multiply itself in several type of cells. Indeed, stem cells can differentiate themselves in osteogenic cells (osteoblasts), cartilage cells (chondrocyte), etc. as it can be seen in *figure 2.9* but in the case of the RGD motif, it will enhance the transformation of stem cells into osteogenic one. Concerning the mineralization property, it consists of secretion of mineral ions such as calcium in the ECM so cells can bind to each other and proliferate. [29] [30] [31]

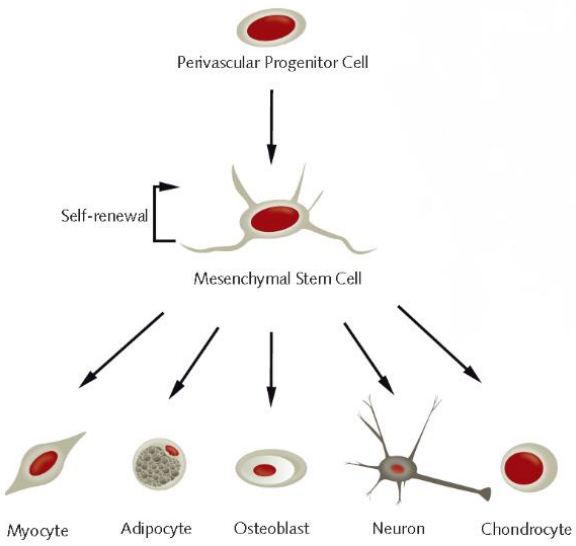

Figure 2.9: Cell differenciation. [32]

The use of RGD peptide in bone tissue engineering comes mainly from its capacity to interact with cell receptors. To summarise, there are several ways for cells to interact. First, they can interact with each other requiring extracellular Ca^{2+} ions that represent the mineralization mentioned previously. They can also interact with each other via different cell receptors like cadherins. However, cells can also interact with the ECM thanks to membrane receptors, of which the major class are the integrins family. They can be either in a resting state or activated state and it is this last state that will enable the interaction between cells and the ECM. They play a significant role in immune response and wound healing. Concerning RGD motif, it is a motif present a large number of NCP of the ECM such as fibronectin, osteopontin or vitronectin. These proteins have the ability to bind to integrins via the RGD motif and therefore promote cell adhesion to the ECM, as it is shown in *figure 2.10*. In other words, RGD helps the ECM to be recognised by cells and this substantial advantage makes this peptide very useful in bone tissue engineering as it provides interactions of the tissue. [24] [33]

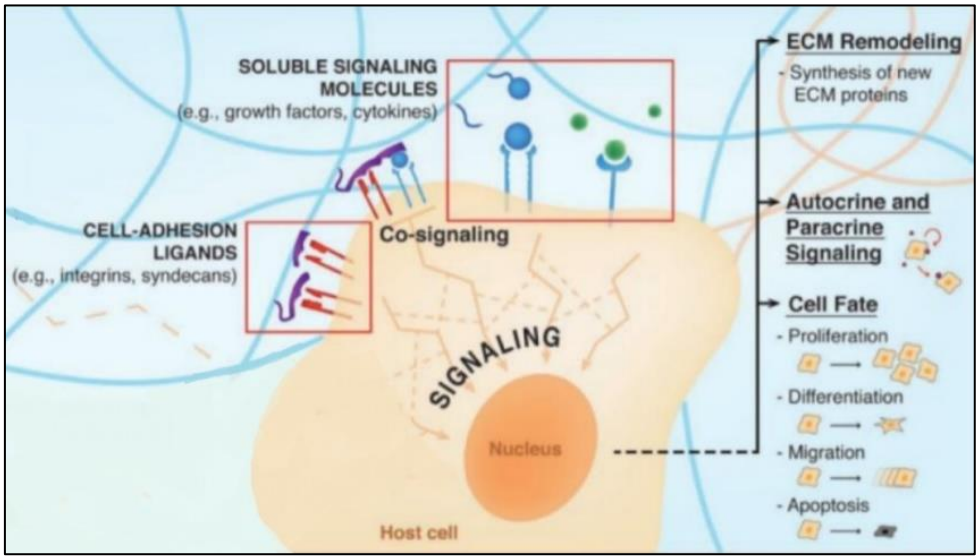

Figure 2.10: ECM and cell interactions. [33]

2.5. 3D-printing / bioinks

3D printing is an additive manufacture developed during the 1980's and consists of printing successive layers of one of multiple materials and deposited on top of each other with a print-head. These materials are printed in the form of inks, mixtures mainly formed by the functional material, a resin and a vehicle. These inks have to flow under moderate pressure and then maintain their shape during and after printing. This process in appearance quite simple enables the creation of really complicated and precise shape pieces. There exist many ways to 3D-print an object such as laser melting or laser sintering but in this project the materials extrusion method will be used. [34]

As it is a quite novel technique, its applications in the biomedical field was quite rare during the last decades. However, it has been growing a lot in recent years especially in TE. Indeed, thanks to its ability to print thin filaments, this process enables creation of precise and complex shapes and geometries but also provide an excellent control of porosity. In the case of scaffolds, porosity and size of pores are really important parameters to control in order to maximize bone formation and enhance vascularization. Moreover, this additive manufacture has also the advantage of being quick and so, many printing tests can be done in a short time. [34]

However, in the case of bone tissue engineering, the use of bioinks is required. Indeed, as the scaffold will be inserted in the human body, the ink will be made to mimic the biological properties of the target tissue but also its mechanical properties. Therefore, bioinks will be used and are defined as inks containing biomaterials and living cells introduced before the printing step. Hydrogel-based bioinks that will be used in this project are typically biocompatible, biodegradable and have cell-binding sites in order to enhance attachment, differentiation and proliferation. They are in general made of biomaterials such as natural polymers (alginate, gelatin, collagen, hyaluronic acid, etc.) but synthetic ones are also used (PEG, PU, polyacrylamide). [35]

2.6. Objectives

The main objective of this project is to develop RGD-functionalised alginate bioinks for bone tissue regeneration. It will be divided on 2 parts:

The first is the functionalisation of the alginate with RGD motif peptide. To do it properly, the functionalisation protocol will be done several times and improved thanks to data found in the literature and results of physico-chemical (FTIR, XPS, Confocal Microscopy, etc.) and biological (Cell culture: attachment and proliferation) characterisations that will be carried out on 2D biofilms to evaluate the efficiency of the crosslinking protocol and verify the presence of RGD peptide

The second consists of preparing and optimizing bioinks for 3D-printing. In other words, incorporating cells in the hydrogel and then printing scaffolds before the last step of physic-chemical / biological characterisation.

RGD crosslinked alginate was studied by several researchers during the 21st century. Finally, printing the combination of alginate and RGD-peptide was only studied in 2014 by Jia Jia et al., however the scaffold developed were made in 2D as it can be seen in *figure 2.11*, deposed onto a calcium substrate in order to crosslink the bio ink but without using a 3D-printer. [36]

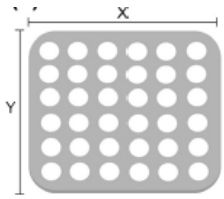

Figure 2.11: shape of the scaffold made in 2014. [36]

As 3D-printing is a quite novel process invented in the beginning of the century, the interest of the study will be to succeed creating RGD-peptide crosslinked alginate bio inks, but this time, printing the material in 3D to create a scaffold that can be inserted in the human body in order to provide bone regeneration.

3. Methods

3.1. Alginate functionalisation with RGD-peptide

3.1.1. First protocol

The first step of the ink preparation is to crosslink the alginate with the RGD-peptide thanks to the EDC/NHS chemistry with the reaction previously shown in *figure 2.7*.

- The alginate used in this project is a powder of sodium alginate medium viscosity with a monomer molecular weight of 198,1 g/mol that leads to chains from 10 000 g/mol to 600 000 g/mol. That alginic acid sodium salt used comes from the brand Panreac.

- The peptide is an *H-Gly-Gly-Gly-Gly-**Arg-Gly-Asp**-Ser-Pro-OH* motif with a molecular weight of 758,74 g/mol per motif and a chemical formula of $C_{28}H_{46}N_{12}O_{13}$. The sequence of 4 glycine is used as a spacer in order to promote attachment and *Ser-Pro* is a sequence that appear many times in proteins such as fibronectin, so it enhances the activity and the attachment to the ECM.

- The Phosphate Buffered Saline (PBS) solution comes from a tablet of Gibco brand dissolved in 500mL of distilled water.

To functionalise alginate, the protocol used is the one developed by Enrique de La Vega in a previous study: [28]

1 – Dissolve 0,08 g of alginate medium viscosity in 7,5 mL of a PBS solution. (10,7 $g.L^{-1}$ / 4,05.10^{-4} mol / 0,054 $mol.L^{-1}$)

2 – Adjust the pH~6 with a solution of HCl 0,25M (diluted from Hydrochloric Acid 37% PA-ACS-ISA Panreac).

3 – Add 10 µL of a 0,25M EDC solution (2,5.10^{-6} mol) from Sigma Aldrich brand and stir during 15 mins at room temperature.

4 – Adjust pH>7 using a solution of 0,25M NaOH (diluting ACS, ISO pallets from Panreac brand).

5 – Add 10 µL of a 0,375M NHS solution (3,75.10^{-6} mol) from Sigma Aldrich brand and stir during 2h at room temperature.

6 – Add 220 µL of a 2,267 mg/mL (0,5 mg / 6,6.10^{-7} mol) peptide solution and stir overnight.

7 – Dialyse for 3 days using a dialysis tubing cellulose membrane D-9777 (cut-off 14000 g/mol) to remove all unreacted reagents. Put de membrane in a 2L beaker and stir the water with a magnetic stirrer. Change water every 2h. (Ref figure 3.1)

8 – Transfer the solution in a plastic container and freeze it using liquid nitrogen.

9 – Lyophilize for several days (until the product is dry).

Figure 3.1: Dialysis of the peptide-alginate solution

Figure 3.2: Functionalised alginate powder

And the amount of reactants chosen is the following according to data found in literature:
The amount of EDC added was such that 0,61% of the carboxylic acid groups were activated
The ratio EDC/NHS is 1:1
The amount of peptide added is 6,25 mg of peptide per gram of alginate

At the end, the product obtained is a sort of foam that can be seen in *figure 3.2*.

3.1.2. Final protocol

After some physical and chemical characterisations carried out that will be explained later, the results revealed an underperformance of the crosslinking. That's why the protocol was improved thanks to some results found in the literature and especially by Mooney et al. [29]

1 – Dissolve 0,08 g of alginate medium viscosity in 7,5 mL of a PBS solution.
(10,7 g.L^{-1} / 4,05.10^{-4} mol / 0,054 mol.L^{-1})
2 – Adjust the pH~6 with HCl 1M.
3 – Mix 82 µL of a 0,25M EDC solution (2,05.10^{-5} mol) with 136 µL of a 0,375M NHS solution (5,10.10^{-5} mol) and stir during 15 mins.
4 – Adjust pH>7 using a solution of NaOH 0,25M.
5 – Add 220 µL of a 2,267 mg/mL (0,5 mg / 6,6.10^{-7} mol) peptide solution and stir overnight.
6 – Dialyse for 4 days using a dialysis tubing cellulose membrane D-9777 (cut-off 14000 g.mol^{-1}) to remove all unreacted reagents. Put de membrane in a 2L beaker and stir the water with a magnetic stirrer. Change water every 2h. Ref figure 3
7 – Transfer the solution in a plastic container and freeze it using liquid nitrogen.
8 – Lyophilize for several days (until the product is dry).

And the amount of reactants chose is the following according to data found in literature:
The amount of EDC added was such that 5% of the carboxylic acid groups were activated
The ratio EDC/NHS is 1:2,5
The amount of peptide added is 6,25 mg of peptide per gram of alginate

The reaction occurring between the alginate and peptide was previously showed in *figure 2.7*. Concerning the pH, a low pH is first introduced in order to enhance the activation reaction with EDC and Sulfo-NHS. Moreover, this low pH is also used because NHS has a low half-life in basic pH (4-5 hours at pH 7, 1 hour at pH 8). Then, the pH is increased since the reaction between Sulfo-NHS and primary amines, such as our peptide, are the most efficient between pH=7 and pH=8.

NB: These 2 protocols were carried out using RGD peptide but also a fluorescent one in order to characterise the functionalisation thanks to confocal microscopy.

3.2. 2D-Films protocol

3.2.1. First protocol

To characterise the functionalisation previously done, the use of films is required in order to carry XPS, confocal microscopy and cell culture characterisations. Therefore, assays of film making protocols were carried out with only alginate in order to improve the quality of films without wasting expensive peptide. Therefore, 1,5g of alginate was mixed in 50mL of water and introduced in the SpeedMixer™ (DAC 150.1 FVZ) for 3 times 5 min at 3500 rpm. After that, 1,5mL of this 1,5% w/v alginate solution was dropped off in a 9cm diameter petri dish plate and then crosslinked 15 mins with calcium chloride 150mM (110,98g/mol Sigma-Aldrich) in order to form the film. Finally, the films were washed with PBS (3 times) in order to remove the remaining ions before cutting and drying them.

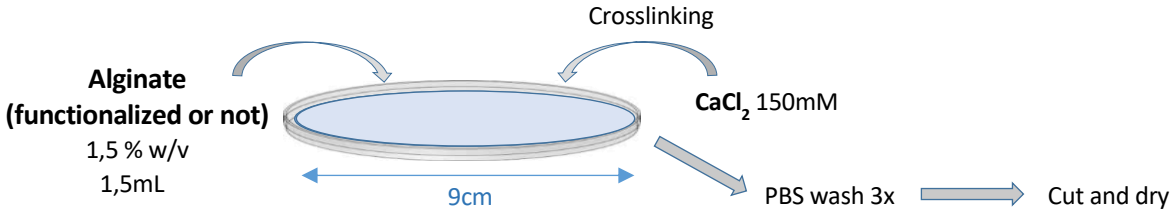

Figure 3.3: Scheme of the preparation of films. Protocol 1.

3.2.2. Improved protocol

As some problems were shown such as break or degradation of films during films manipulation, some modifications of the protocol were tested such as increasing the crosslinking time or increasing the volume added in the petri dish plate (2,5mL / 5mL / 10mL) to get thicker films. Also, the weight concentration was changed (3% w/v) and assays controlling water evaporation before the crosslinking step in order to get denser films were carried out.

At the end, the final protocol chosen consists of putting 5mL of a 3% w/v alginate solution (3g of alginate in 50mL of water introduced in the SpeedMixer™ (DAC 150.1 FVZ) for 3 times during 5 min at 3500 rpm) in a 9 cm diameter petri plate. Then, the alginate is crosslinked during 1h with $CaCl_2$ 150mM. Finally, the films were washed with water (PBS degrades the films) for 3 times before cutting and drying them.

3.3. CELL CULTURE

The cell culture is the most important characterisation of this project as it enables to quantify the bioactivity of the scaffold and the presence of peptide. The protocol is the following:

I) CLEANING. First, cleaning the hood and the materials with ethanol is necessary in order to avoid contamination. Ethanol is also used to sterilize the films before starting any other manipulations.

II) PREPARATION OF THE MEDIA. Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM) is mixed with a buffer: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to maintain physiological pH. Then L-glutamine and penicillin antibiotics are added to prevent bacterial infections. Moreover, 2 medias are elaborated, one with Fetal Bovine Serum (FBS) to enhance attachment and one without. The recipes are the following:

DMEM – 43mL *(86%)*	**DMEM** – 48mL *(96%)*
FBS – 5mL *(10%)*	**HEPES** – 1mL *(2%)*
HEPES – 1mL *(2%)*	**L-glutamine** – 0.5mL *(1%)*
L-glutamine – 0.5mL *(1%)*	**Penicillin** – 0,5mL *(1%)*
Penicillin – 0,5mL *(1%)*	

III) PREPARATION OF CELLS. As MG63 cells are agglomerated at the bottom of the falcon, the media is taken off. Then, a rinse with PBS is required to take off completely the media and 2mL of trypsin is used to detach the cells. However, as trypsin is active only at 37°C, it had to be put in the incubator. After that, the trypsin is neutralised with 4mL of media with FBS and taken off after 5min centrifugation at 300 Relative Centrifugal Force (RCF).

IV) CELL COUNTING. For this step, 3mL of each media is mixed with cells thanks to a vortex. Then, 10 µL of these solutions is taken off and put on a Neubauer chamber. After counting thanks to a microscope, the media is once again diluted depending on the previous counting, in order to obtain a concentration of 50 000 cells per mL.

Then, the next steps depend on the type of cell culture wanted: attachment or proliferation

ATTACHMENT	**PROLIFERATION**
V) CELLS SITTING. 0,5mL of media is added in the plate for each film (25 000 cells). Then, they are kept in the incubator (37°C) for 6h.	**V) CELLS SITTING.** 0,5mL of media is added in the plate for each film (25 000 cells). Then, they are kept in the incubator (37°C) for 4h. After that, the media is removed and an Alamar blue cell viability agent 1:10 Media solution is added at a quantity of 350 µL for each film for 1h.
VI) FIXATION OF THE CELLS. Films are washed 1 time with PBS and then are fixated with 300 µL of a 4% solution of Perfluoroalkoxy Polymer (PFA) during 15 mins. Finally, films are washed 3 times with PBS.	**VI) CALIBRATION CURVE.** In order to interpret the results and correlate number of cells and fluorescence, a calibration curve is required. To do so, the cells sitting solution is added in several plates with different number of cells (2 500 to 80 000) at an amount of 350 µL and kept during 1h30 in the incubator. Then, the plates are put in a BioTek plate reader (model Synergy HTX) to quantify
VII) PERMEABILISATION. Alexa fluor 546 phalloidin is mixed with a permeabilization solution (triton X-100 1:2000 PBS) in 1:300 proportion. The PBS is removed and 250 µL of this solution is added in the plate of each film during 1h.	

The phalloidin will stain the betectine filament (cytoskeleton) of the cells. But as it is a big molecule, permeabilization with triton is required so it can pass through. Concerning alexa fluor, it is used to see the cells at the confocal.

VIII) FLUORESCENCE OF THE NUCLEI. 250 µL of DAPI solution is added in each plate containing a film which is basically a solution of DAPI 1:1000 washing solution (glycine 1:666 PBS)

Finally, films are ready to be observed at confocal microscopy to be characterised.

the fluorescence and correlate it with the cells number. Then, the curve can be drawn.

VII) RESULTS. To obtain the results, the plate is stocked in an incubator and put each day in the plate reader to know the fluorescence and so, thanks to the calibration curve, know the number of cells and their proliferation rate. The results are converted in number of cells per mm² because several diameters were used:

Cover sleep	D = 11,7 mm
Alginate	D = 6,3 mm
RGD-Alginate	D = 6,3 mm

3.4. FILAMENTS PROTOCOL

In order to characterise the presence of peptide, confocal microscopy is used on films made of crosslinked alginate with fluorescent peptide. But filaments are also made to see the homogeneity and the repartition of this peptide in a 3D shape. To do so, 0,5g of a 3% w/v crosslinked alginate solution is introduced in a Nordson EFD syringe. The solution was previously made introducing the mixture in the SpeedMixer™ for 3 times 5 min at 3500 rpm. The needle is then pressed and the filament pass through a Norson EFD .016'' (406,4µm) nozzle directly in a solution of 150mM calcium chloride to form the filament. In the meantime, spinning the syringe is required to avoid agglomeration as it can be seen in *figure 3.4*.

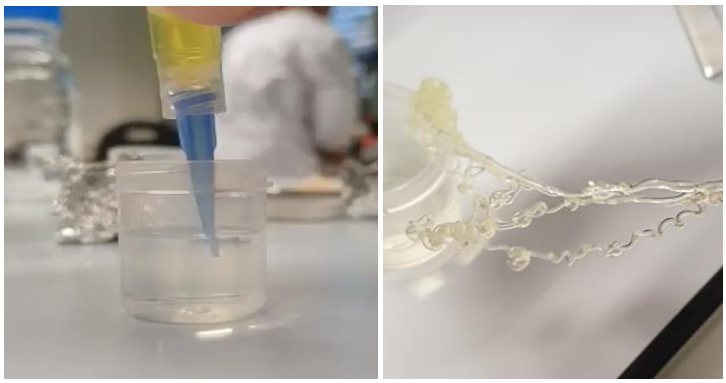

Figure 3.4: Preparation of alginate filaments.

The filament made of fluorescent peptide is then hidden from the light until the characterisation and the same will be done for a non-crosslinked alginate filament so it can be compared.

Alginate and functionalised alginate filaments filled with cells were also elaborated:

- First, the powder and all toolds used such as syringes, nozzles and pots were sterilized with ethanol.

- Two pots were filled with 750µL of media and with respectively 0,03g of pure alginate and 0,03g of functionalised alginate.

- The solutions are mixed thanks to the speed mixer (3 times 5 minutes at 3500 rpm).

- 250µL of media is then added to each pot with 1 million cells inside. The cells were before counted thanks to a Neubauer chamber and then diluted to obtain the correct amount. After that, the mixture is mixed by hand to keep cells alive.

- Finally, solutions are put in syringes and pressed into a calcium chloride (150mM) bath to be crosslinked.

Then, cells need to be stained in order to carry out a life/dead assay. To do so, a staining solution is elaborated using 3,5mL of DMEM, 10,5µL of calcein solution (green staining for all cells) and 3,5µL of propidium iodide solution (red staining for dead cells). 0,5mL of staining solution is put in each plate during 5 minutes. Finally, the samples are ready to be analysed with LCSM at day 0,1,3,7 and 14, keeping them in the incubator between each characterisation.

3.5. CARACTERISATION METHODS

3.5.1. Fourier Transform InfraRed (FTIR)

Fourier Transform InfraRed (FTIR) spectroscopy is a technique that enables to analyse solids (powder, film, etc.) and liquid samples and identify their functional groups. Basically, a polychromatic infrared beam is introduced onto an optically dense crystal (Germanium) that reflect the infrared beam onto the samples and thanks to their different absorbance and transmittance properties at different wavelengths, an IR spectrum is obtained. This spectrum shows the transmittance of the material according to the wavenumber (cm^{-1}) and so enables to determinate the molecular composition and structure of the material according to the wavenumber at which are located the peaks and the tables found in the literature. [37]

The FTIR equipment used in is the spectroscope Thermoscientific Nicolet 6700 and the acquisition of the data on the OMNIC software. In this case, the characterisation of pure alginate and functionalised alginate powder after freezing and lyophilisation were carried out using a resolution of 2 and 512 scans.

3.5.2. X-Ray Photoelectron spectroscopy (XPS)

X-Ray Photoelectron spectroscopy (XPS) is a photoelectric spectroscopic and non-destructive technique that enables the measurement of the material elemental composition. The XPS spectrum is drawn after irradiating the material with an X-rays beam. Indeed, some electrons on the surface of the materials will be emitted and their emission is analysed. The electrons ejected are counted according to a range of electron kinetic energies. The peaks will then enable to identify the surface elements. It is a good way to identify the chemical composition of the samples as all type of materials can be used for this characterisation. Moreover, this method enables also to analyse the empirical formula, chemical state and electronic state of the sample's elements. However, a drawback is that only the surface of the material can be scanned (up to 10nm). Therefore, the results found are likely not similar to the one in the bulk. Also, a vacuum is required. [38]

The equipment used in this case is from SPECS Brand (Berlin, Germany) with as a source: a XR50 dual anode (only the Al anode operating at 200W was used) and as detector: the Phoibos 150 MCD-9 using a pass energy of 20eV.

The parameters used were: - Vacuum: under 5x10-9 mbar

- Survey: 0.1eV/step, 3 scans
- High resolution spectra: 0.05eV/step, 5 scans

3.5.3. Laser Confocal Scanning Microscopy (LCSM)

Laser Confocal Scanning Microscopy (LCSM) is a technique widely used to detect fluorescence. The difference with other fluorescence techniques is its ability to detect only fluorescent light that is close to the focal plane thanks to a focused laser beam that produces spot illumination on the sample, this is why it is possible to remove the background glow and so having great quality and resolution of images. At the end, by combining every optical plane, it is possible to produce a 3D image. This technique is mostly used in biology and medicine to evaluate several eye diseases or to detect molecular, cells and proteins interactions. Finally, no sample preparation is required but for high resolutions such as 63X, oil immersion is required. [39]

Fluorescence images were obtained with the equipment Zeiss LSM 800 using 5x, 10x and 63x objectives (the higher one requiring oil immersion). The acquisition of images was done using Zen 2.3 software from Zeiss company and modified using ImageJ software.

4. RESULTS AND DISCUSSIONS

4.1. 1st functionalisation protocol

4.1.1. FTIR

As it was previously said, the first protocol carried out was the one used by a previous student Enrique de Vega GOMEZ, the protocol is explained in the method part. [28] After the last step of lyophilisation, the powder collected has been compared to pure alginate thanks to FTIR characterisation, the results are shown in *figure 4.1* and *4.2*.

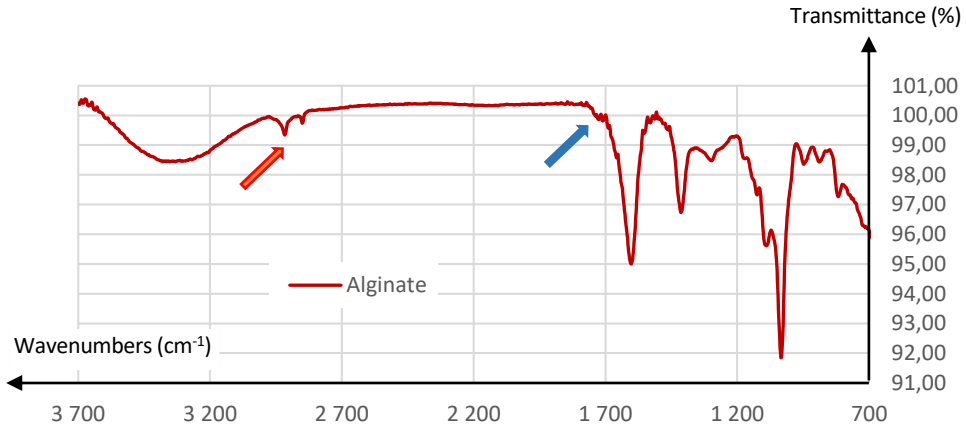

Figure 4.1: FTIR spectrum of alginate.

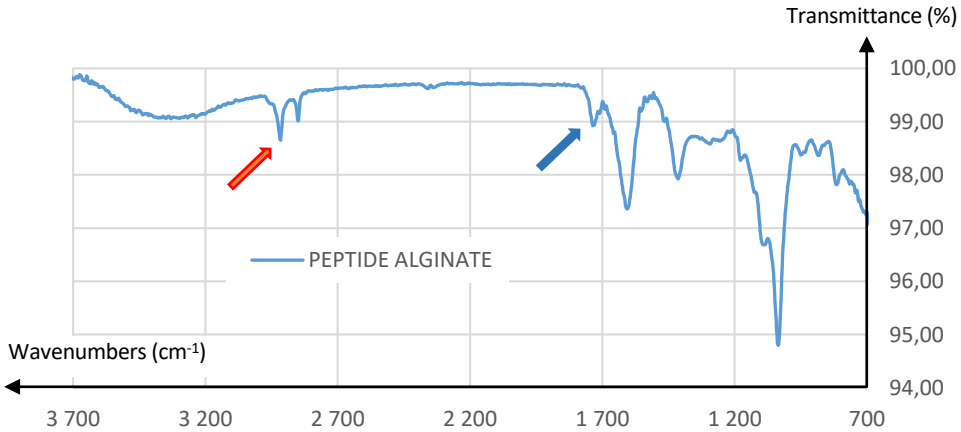

Figure 4.2: FTIR spectrum of functionalized alginate from 1st protocol.

The FTIR did not show any significant results. Indeed, only small changes in the spectrum could be noticed such the peak of the C-H bond (2850-3000 cm^{-1}) shown with red arrows that is more distinct for the functionalised powder (*fig 4.2*) than the pure alginate (*fig 4.1*). Moreover, a C=O peak (1750-1800) shown with blue arrows can be recognised in *figure 4.2* and cannot be seen with pure alginate. These results

show a difference in the molecular architecture between the 2 powders. However, the difference of peak intensity is quite small and only a few percentages of transmittance differ. This is why it is impossible to conclude about the performances of the crosslinking without carrying other characterisation techniques such as a biological one.

4.1.2. Cell culture

Another characterisation method carried out is cell attachment. After achieving the elaboration of 2D biofilms according to the first protocol, a cell adhesion protocol was carried out on functionalised alginate films, alginate reference films and coverslips using media with and without FBS. But once again, the results were mixed. Indeed, some cells were attached on the bottom but almost none on the top of the films. There were no significant differences between functionalised and non-functionalised films and the few cells attached kept a round shape which means that they were badly attached. As a conclusion, almost no bioactivity was observed and these results lead to the fact that there is a problem in the functionalisation and show an insufficient quantity of peptide in the biofilm. That's why the first functionalisation protocol has been improved into the final one thanks to results found in the literature.

Another problem of this cell culture assay was the difficulty to manipulate the films and sometimes their degradation. That's why it was impossible to take pictures of this assay. At the end, the first film elaboration protocol also needed to be improved into the final one shown in the method part in order to achieve making stiffer films that would be easier to manipulate and so to characterise.

4.2. 2nd functionalisation protocol

4.2.1. FTIR

FTIR technique was also carried out for the final protocol, the results found are shown in *figure 4.3* and *4.4.*

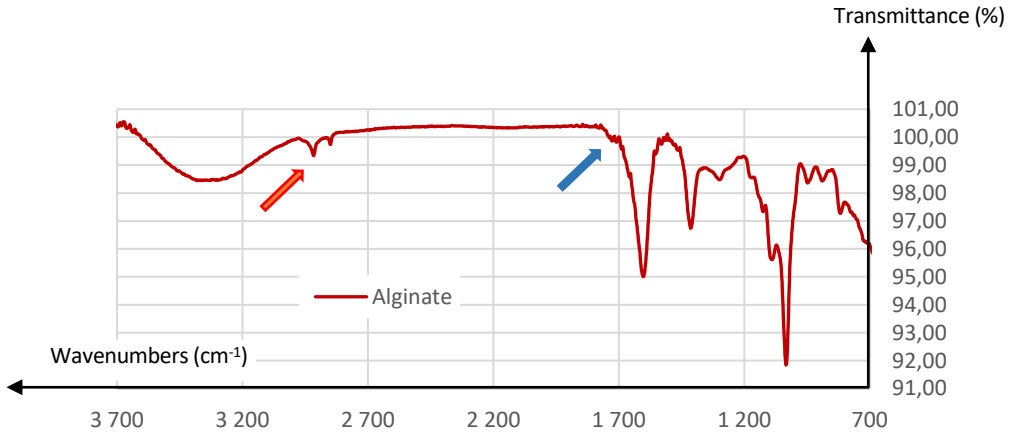

Figure 4.3: FTIR spectrum of algina

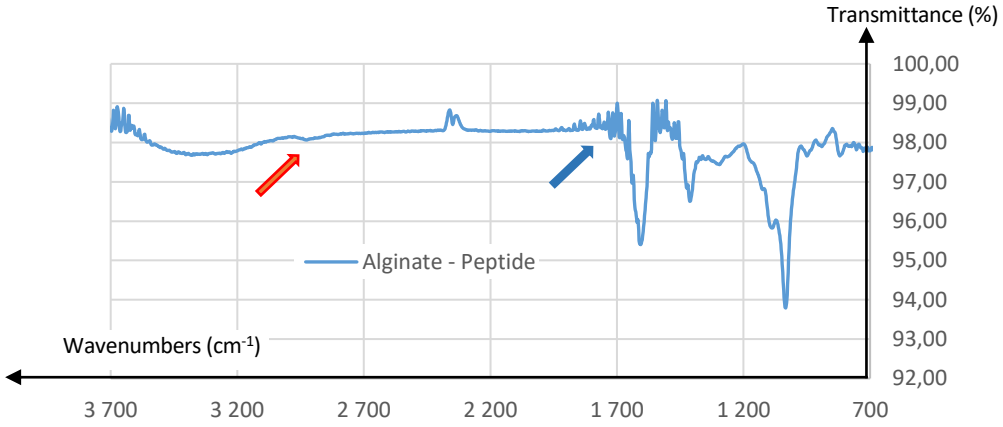

Figure 4.4: FTIR spectrum of functionalized alginate from 2nd protocol.

Once again, no significant result was found. Indeed, the small C-H bond (2850-3000 cm^{-1}) shown with red arrows completely disappeared in *figure 4.4*. In addition, the C=O peak (1750-1800) shown with blue arrows was present in the spectrum of the functionalised alginate from the first protocol (*fig 4.2*) but is here quite hard to recognise. At the end, the conclusion is similar to the last FTIR characterisation, a molecular structure change is recognised but the differences being too little, it is impossible to draw conclusions of this assay. Indeed, either the functionalisation could have not worked or the amount of peptide could be too little compared to the amount of alginate so the difference would be hard to seen with FTIR. This is why other characterisation techniques are required.

4.2.2. XPS

XPS was carried out using 2 functionalised films and 2 references made with pure alginate. The elements of the analysis included C, O and N. The analysis of the N signal was particularly interesting as this element is only found in the peptides. However, as detailed in Table X, no differences were observed between alginate and alginate functionalized with RGD in the chemical composition at the atomic level. As explained in the previous characterisation FTIR, the amount of RGD peptide used is very low compared to alginate. Therefore, it is complicated to detect any composition differences as it can be seen in *figure 4.5*. The table used to draw this graph can be found in annex (*table A*).

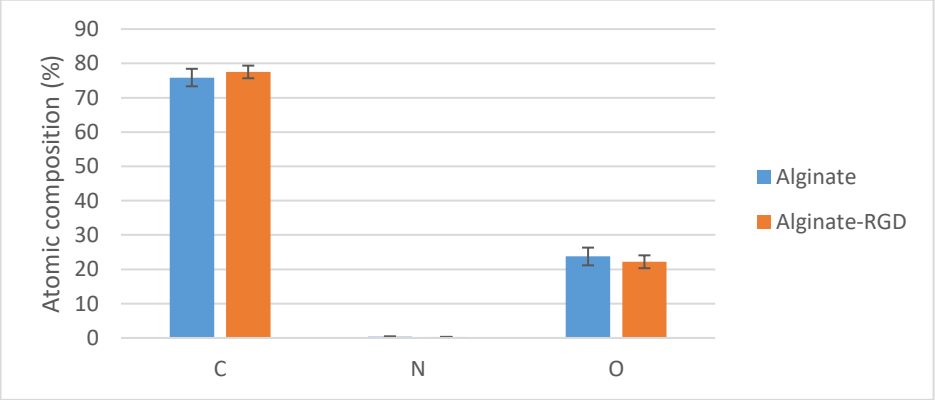

Figure 4.5: Graph of XPS results showing films atom composition.

However, significant differences were found when the C1s peak was deconvoluted into its main components, as it can be seen in *figure 4.6*. The table used for this graph is in the annex part (*table B*).

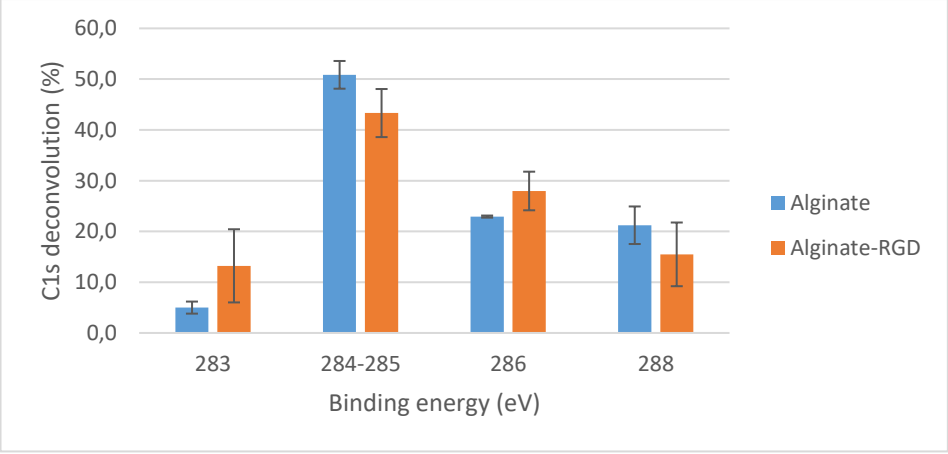

Figure 4.6: XPS results graph for the deconvolution of the C1s peak at different binding energies.

Interestingly, quantitative changes are noticeable in the composition of the C1s components, as evidenced by the different % obtained for the distinct binding energies of C1s. This means that even if the peptide cannot be detected, its presence changes the chemical architecture of alginate. Thus, XPS results indirectly support the success of the grafting procedure.

4.2.3. LCSM

4.2.3.1. 2D films

As FTIR and XPS results didn't put in evidence the presence of peptide, the crosslinking protocol was also carried out with fluorescent peptide. Then 2D biofilms were made in order to be characterised by confocal microscopy. The results are shown in *figure 4.7*.

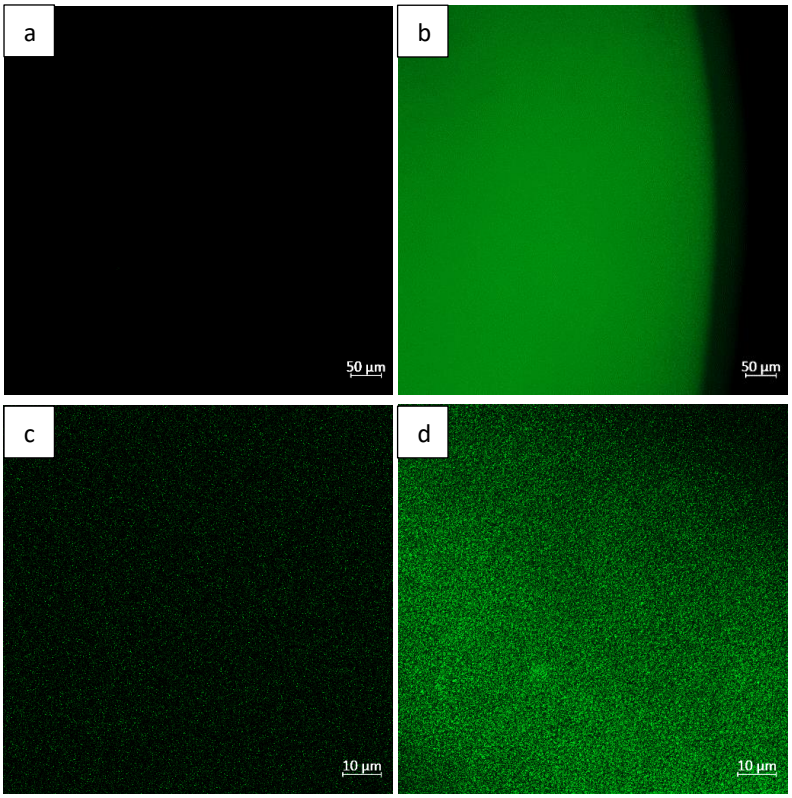

Figure 4.7: CLSM images of 3% w/v films of alginate at 10x (a), 63x (c) and Fluorescent RGD functionalised alginate at 10x (b) and 63x (d)

With the purpose of analysing the presence of RGD, green fluorescence and borders delimitations of *figure 4.7(b)* show the presence of peptide. As the reference *(a)* show no fluorescence, the results shown do not come from background but from a good functionalisation of the alginate with the RGD motif. The assay also shows a much higher fluorescence of the functionalised alginate *(d)* at higher magnitude than non-functionalised alginate *(c)*. Concerning the green points present in figure 4.7*(c)*, they come from background fluorescence.

4.2.3.2. 3D filaments

Even if the results of this LCSM assay were quite encouraging, the same characterisation was carried out on 3D filaments to be sure of the homogeneity and the repartition of the peptide in the hydrogel. The results found are shown in *figure 4.8.*

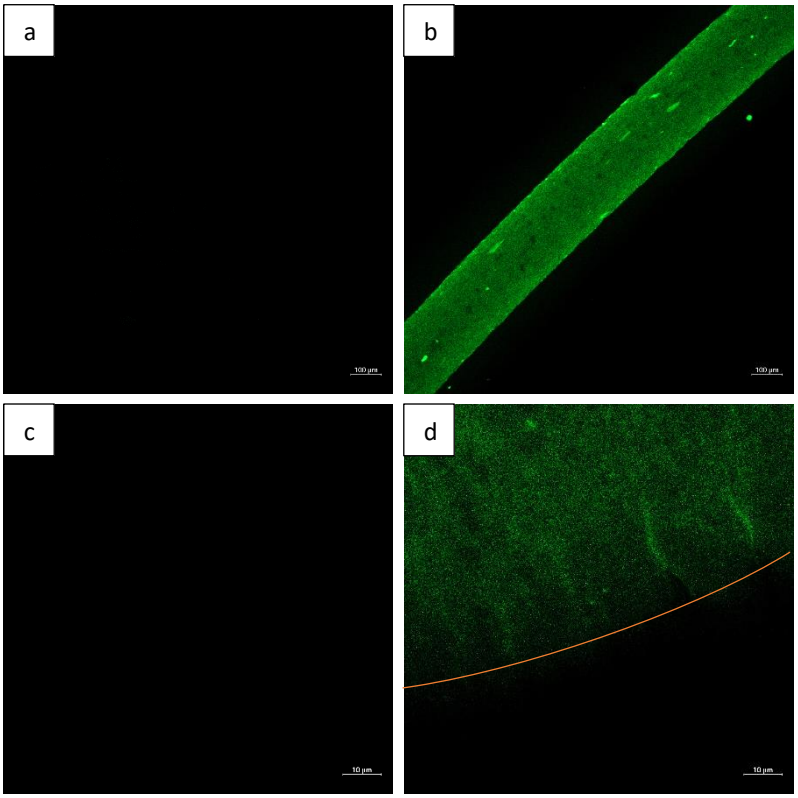

Figure 4.8: CLSM images of 3% w/v filaments of alginate at 5x (a), 63x (c) and Fluorescent RGD functionalised alginate at 5x (b) and 63x (d)

At low magnitude, it is clear that RGD functionalised filament (*fig 4.8(b)*) is much more fluorescent than the alginate one (a) and so contain the fluorescent peptide. Moreover, even though some agglomerations can be seen at different places of the sample, the repartition of the peptide seems quite homogeneous. Same results were found at 63x magnitude, alginate filament (c) appears without any fluorescence whereas the functionalised one (d) show clearly fluorescence. Also, the border of the filament is well defined as it can be seen in *figure 4.8(d)* by the drew orange line.

4.2.4. Cell culture on 2D films

As the last assay proved the presence of peptide in the hydrogel after the functionalisation protocol, it is necessary to verify its bioactivity thanks to biological cellular assays.

4.2.4.1. Cell attachment assay

The first assay carried out was a cellular attachment explained in the method part to compare functionalised alginate with non-functionalised one. The 2D films were made using the improved protocol.

4.2.4.1.1 Optical Microscopy

After sitting cells (**step V**), which consists of their deposition and their adhesion on films, optical microscopy was carried out and the images obtained are shown in *figure 4.9*.

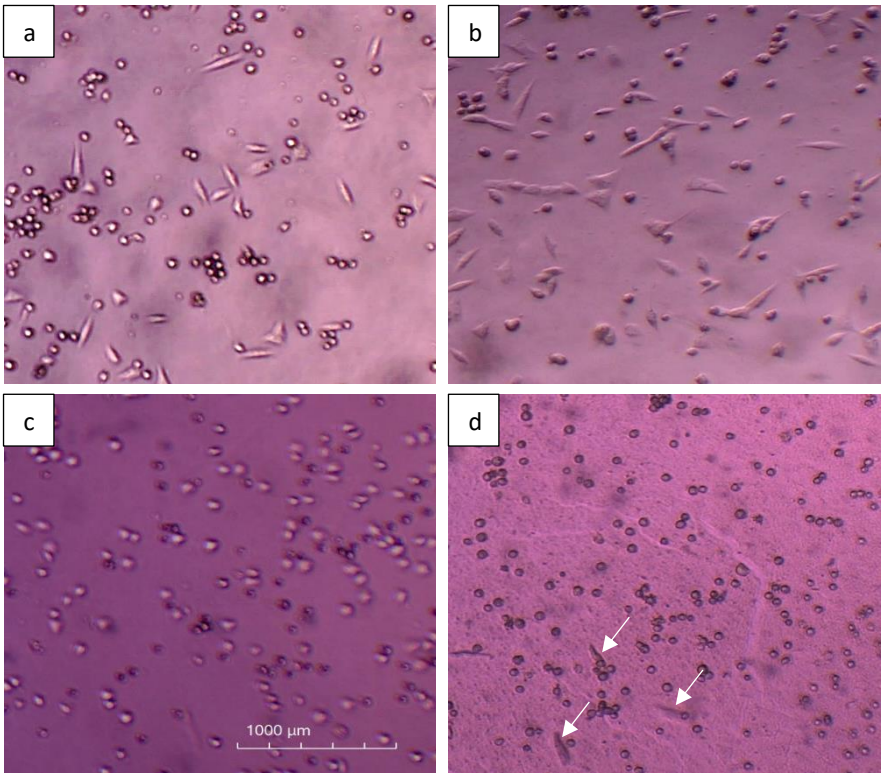

Figure 4.9: MG63 cells adherent to control alginate surface (a) and RGD modified alginate surface (b) with FBS and respectively (c) and (d) without FBS at 6h post-seeding.

In *figure 4.9.a* and *4.9.b*, cells are much more spread and this is due to participation of FBS that possesses cell proliferation properties as it provides many nutrients and growth factor that enhance

their proliferation. Moreover, without FBS, it is clear that more cells have spread and *so* proliferated on functionalised alginate surfaces (*a*) than on non-functionalised ones (*b*). The difference is also noticeable without FBS; indeed, some spread cells can be seen on RGD-modified alginate (*d*) and are pointed by white arrows whereas none are distinct on alginate films (*c*).

As a conclusion of this assay, the results seem quite encouraging and confirming a better bioactivity of functionalised alginate. To confirm these results, LCSM technique is then used.

4.2.4.1.2 LCSM

After step **VIII** of the cell culture attachment protocol, cells are already fixed and fluorescent thanks to Alexa Fluor and DAPI for the nuclei, it enables their visualisation in a confocal microscopy assay. The pictures taken thanks to LCSM are shown in *figure 4.10*.

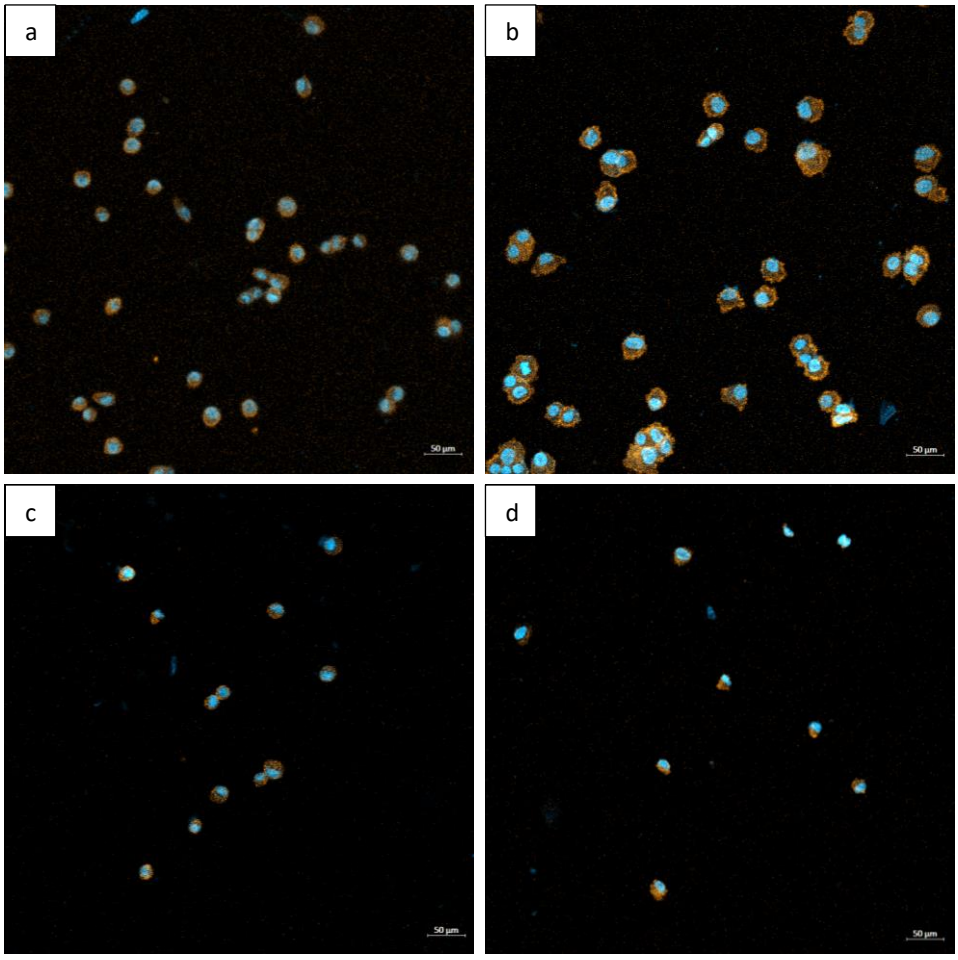

Figure 4.10: CLSM images of 3% w/v films of alginate (a) and RGD modified alginate (b) with FBS And respectively without FBS (c) and (d) at 6h post seeding.

In these pictures, blue colour comes from DAPI and represents nuclei of cells and orange/red colour comes from Alexa fluor and represents the betectine filaments (cytoskeleton) of cells. As in optical microscopy pictures, cells are much more numerous and spread with FBS (a,b) compared to without (c,d). An aspect of these results is that, without FBS, no major differences can be recognise between functionalised alginate (d) and non-functionalised one (c), the number of cells is quite similar and their spreading seem limited in both figures. However, what is relevant here is the pictures with FBS. It can easily be noticed that cells are much more spread in presence of RGD (b) than without (a). Several cells are agglomerated but even the ones that are far from the other are well spread which means that the surface has bioactive properties and so prove the presence of RGD motif.

At the end, optical microscopy results agree with the LCSM ones and show bioactivity properties of biofilms as expected.

4.2.4.2. Cell proliferation assay

Cell proliferation assay was then carried out using the protocol described in the method parts. The results used to draw the calibration curve represented in *figure 4.11* are shown in annex (*table C-D*).

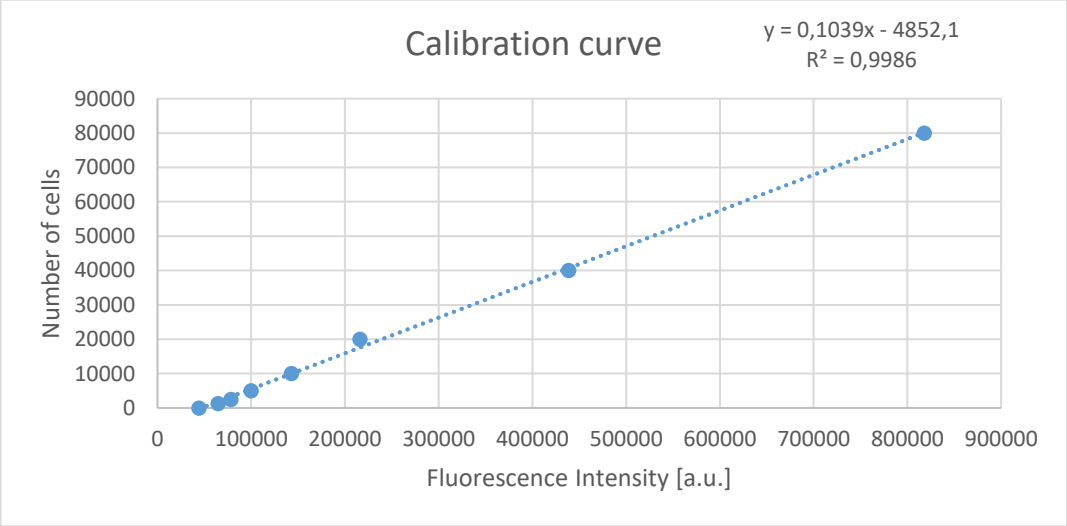

Figure 4.11: Calibration curve of cell proliferation assay.

Then, thanks to this calibration curve, number of cells can be correlated to fluorescence intensity and the following graphs can be drawn at day 1, 2 and 7 after seeding.

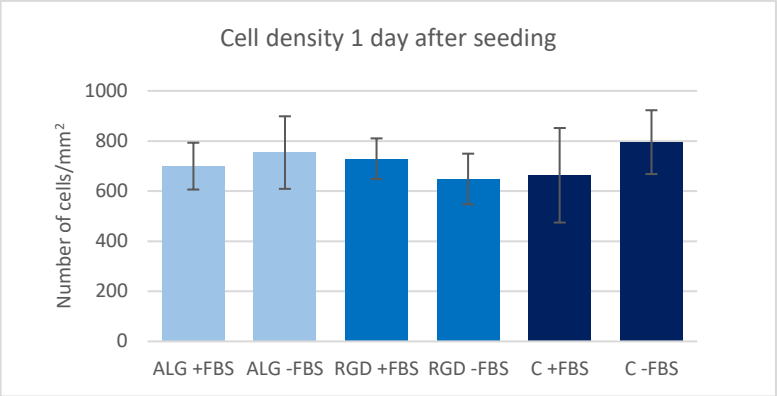

Figure 4.12: Histogram of samples cell density at 1-day post-seeding.

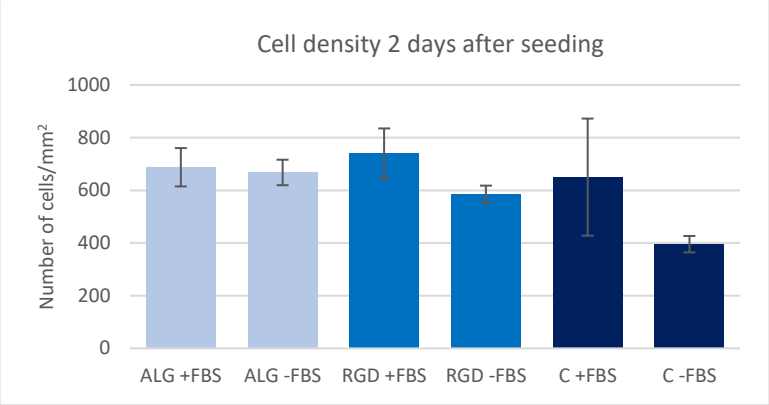

Figure 4.13: Histogram of samples cell density at 2-days post-seeding.

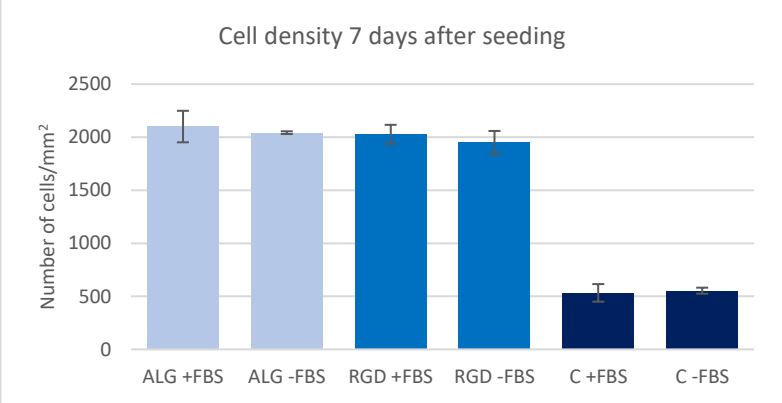

Figure 4.14: Histogram of samples cell density at 7-days post-seeding.

Results of proliferation assay in *figure 4.12*, *4.13* and *4.14* show globally an enhancement of cell density over time for functionalised and non-functionalised alginate films. However, no major difference is noticeable between the 2 samples. Also, presence or absence of FBS do not provide density changes. So, questions concerning the reliability of these experiments arise. Another unusual fact is that a diminution of cell density is detected for cover sleep samples which indicates a death of a high number of cells on control samples. At the end, these results are questioned and an issue in the experimental

condition or a product contamination is suspected. A repetition of this experience is so required to exploit the results but not carried out in this project because of lack of time.

However, apart from proliferation results, even if these biological assays seem quite promising, experiments were carried out on films and so on 2D surfaces of samples. And as this project include hydrogels, they should normally provide abundant space in their 3D-network for cells to grow. That is why biological characterisations have to be carried out on 3D-filaments to confirm biofilms results. However, because of lack of time, it had not been carried out until the end in this study.

4.2.5. Cell culture on 3D filaments

4.2.5.1. Life/dead assay

Life/dead assays were carried out as explained in the methods part and the results at 2h, day 1 and 3 post-seeding are shown in *figure 4.1*.

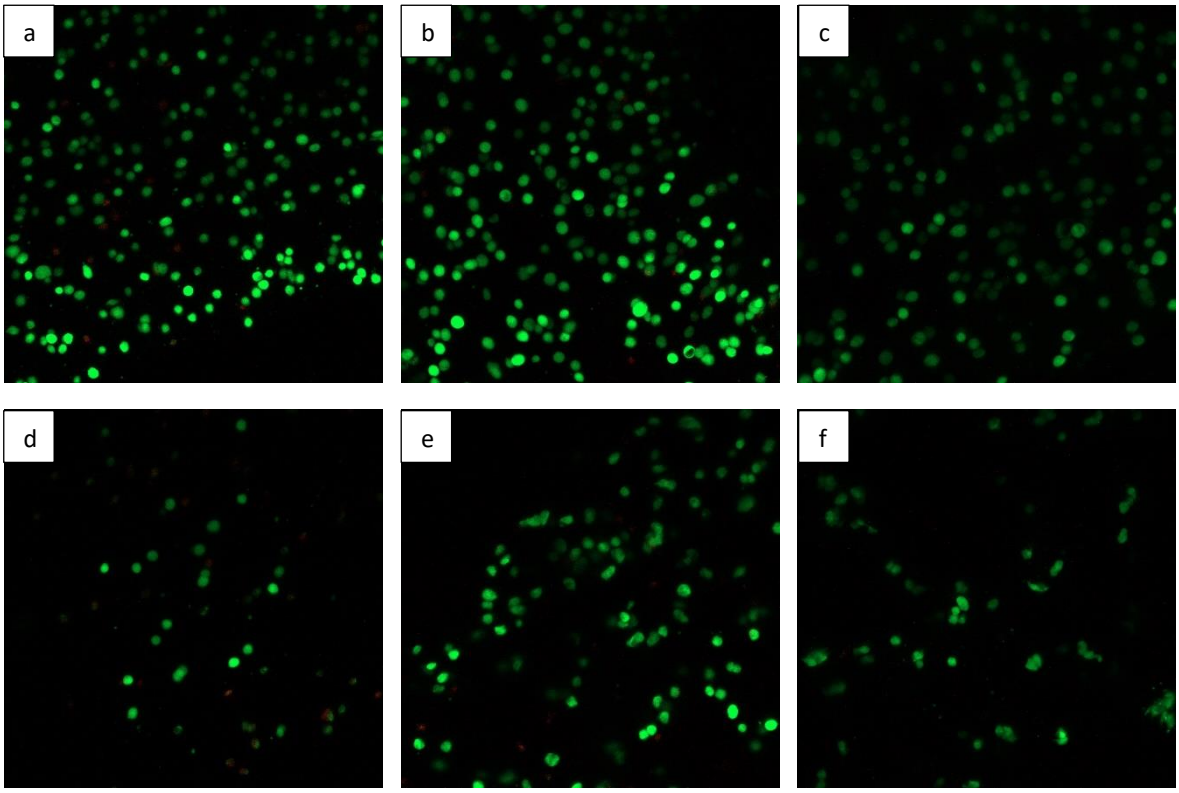

Figure 4.15: CLSM images of 3% w/v films of alginate (a,b,c) and RGD modified alginate (d,e,f) at 2h (a,e), day 1 (b,e) and day 3 (c,f) post-seeding.

As seen in *figure 4.15*, cells seemed viable during the entire incubation period for all conditions. However, the presence of the peptide did not seem to improve the number of adherent cells. On the

contrary, the number of cells seemed to be reduced on RGD-coated samples, compared to control alginate.

These results were not as expected; however, the RGD-coated alginate filaments were prepared from the 2D films previously used during the project and the integrity of the resulting filaments was not optimal. In fact, the filaments did not seem to be strongly crosslinked and were partially disaggregating. Thus, the lack of bioactivity could be partially attributed to this handling problems. More experiments in this regard would be required.

4.2.5.2. Proliferation assay

Proliferation assay was also carried out using the same protocol as for biofilms. The results are shown in *figure 4.16*.

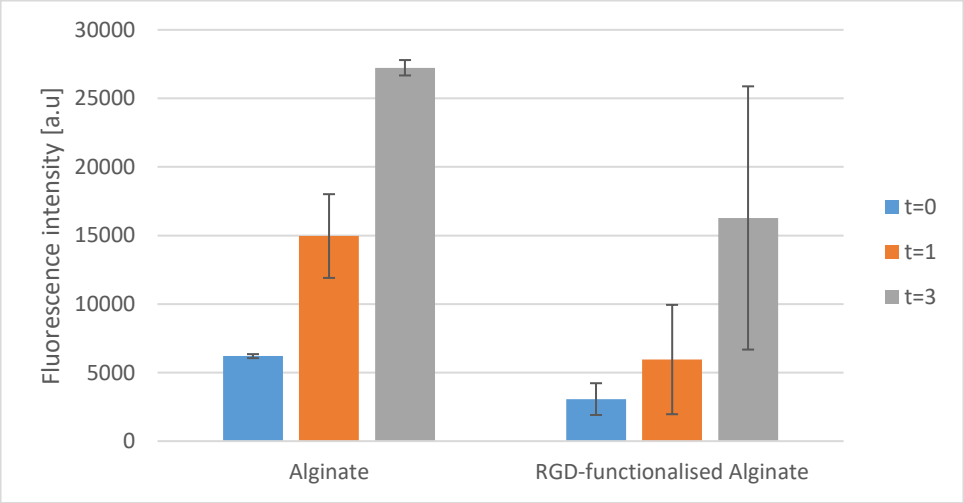

Figure 4.16: Histogram of Fluorescence intensity of pure alginate and functionalised alginate filaments 2h, day 1 and 3 post-seeding.

In agreement with the live / dead experiments, it seems that cells are viable in the filaments and in fact are capable of proliferating. However, the RGD peptide is not showing any positive effects in the growth of the cells. Here, again, the problems previously commented on the integrity of the filaments could be the reason for such outcome. Further work is necessary to explain these effects.

Conclusions

During this project, RGD functionalised alginate bioinks were developed in order to be 3D printed as scaffolds for bone TE. To characterise this functionalisation, 2D biofilms were elaborated and their protocol improved. After carrying assays on these films, difficulties appeared concerning revealing the presence of peptide using physical and chemical characterisations such as FTIR and XPS techniques. However biological results on these films revealed presence and usefulness of RGD motif as a bioactive enhancer especially promoting cell adhesion and cell proliferation. Assays on cell incorporated filaments were also carried out to confirm the hydrogel biological properties at a 3D scale but no promising results were shown as some experimental conditions distorted the assay, such as reusing functionalised RGD. To conclude, the methodology of RGD-modified alginate hydrogel elaboration, previously unknown by the research group, has been established and optimized but still need some improvements.

To continue this project, characterisation tests need to be redone in order to confirm the previous results. But also, further studies carrying other characterisation tests have to be run such as radiolabelling, radioactivity techniques or cell differentiation markers, to specify the biological properties of the future scaffold. Moreover, assays on 3D filaments filled with cells have to be redone. Finally, assays of 3D printing could be run and printing optimisation carried out in order to finish this study.

Economic Analysis

An economic analysis of this project was carried out to estimate its global price. This analysis revealed the amount of 8 495€ spent in this project. The details are shown in the tables bellow:

First, 6 alginate functionalisation protocol have been performed and their cost is shown in *table 1*.

Table 1: Alginate functionalisation.

Product	Quantity	Price	Cost(€)
RGD	3 mg	100€ / mg	300
Fluorescent RGD	1,7 mg	100€ / mg	170
Alginic acid sodium salt	0,72 g	31,40€ / 250g	0,09
Distilled water	40 L	0,60€ / L	24
Dialysis membrane	2 m	132,30€ / 30m	8,82
NHS	0,036 g	685€ / kg	0,022
EDC	0,0245 g	374€ / 25g	0,36
PBS	1 tablet	168€ / 100 tablets	1,68
			504,97 €

Then films protocol was elaborated and improved many times.

Table 2: Film elaboration.

Product	Quantity	Price	Cost(€)
Alginic acid sodium salt	2 g	31,40€ / 250g	0,25
Distilled water	2 L	0,60€ / L	1,20
Calcium chloride	16,5 g	112€ / 100g	18,48
PBS	1 tablet	168€ / 100 tablets	1,68
			21,61 €

3 biological cell attachment and 2 proliferation protocols were then carried out.

Table 3: cell culture.

Product	Quantity	Price	Cost(€)
24-well plate	4 units	65€ / 5 plates	52
15mL falcon	10 units	200€ / 500 units	4
PBS	1 tablet	168€ / 100 tablets	1,68
DMEM	182 mL	25,60€ / 500 mL	9,32
FBS	10 mL	184€ / 100 mL	18,4
HEPES buffer	4 mL	63,80€ / 100 mL	2,55
L-glutamin	2mL	23,30€ / 100 mL	0,47
Penicillin	2mL	66,30€ / 100 mL	1,33
Cells	~ 4 millions	0 (already in the lab)	0
Focal Adhesion Staining Kit (TRITC, phalloidin, DAPI)	4 assays	433€ / 100 assays	17,32
PFA	400 µL	47,10€ / 250 mL	0,08
			107,15 €

Table 4: characterisation techniques.

Equipment	Time (h)	Price (€/h)	Cost(€)
FTIR	3	5	15
XPS	6	80	480
CLSM	8	60	240
			735 €

Table 5: Staff.

Position	Time (h)	Price (€/h)	Cost(€)
TFM Student	720	0	0
Project director	60	60	3 600
Project co-director	30	60	1 800
technician	17	31	527
PhD Student	40	30	1 200
			7 127

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Annex

Table A: XPS results of films atom composition. Data shown as %.

	C		N		O	
	X	SD	X	SD	X	SD
Alginate	75,875	2,552655	0,375	0,035355	23,745	2,58094
Alginate-RGD	77,51	1,838478	0,3	0,028284	22,195	1,873833

Table B: XPS results for the deconvolution of the C1s peak at different binding energies. Data shown as %.

	C 1s 283 eV		C 1s 284-285 eV		C 1s 286 eV		C 1s 288 eV	
	X	SD	X	SD	X	SD	X	SD
Alginate	5,0	1,2	50,9	2,7	22,9	0,2	21,2	3,7
Alginate-RGD	13,2	7,2	43,3	4,7	28,0	3,8	15,5	6,3

Table C: Fluorescence corresponding to number of cells (plate reader results).

Number of cells	Fluorescence intensity [a.u.]								
80 000	764502	769953	763177	904303	888145	882206	796505	800062	793545
40 000	481084	485309	490116	433511	426239	429398	401979	401785	397195
20 000	214718	215486	214404	213022	216678	217379	215458	221610	214216
10 000	143951	145054	145677	148132	149347	148114	134309	135233	135583
5 000	99501	98747	99361	98994	98677	101474	101851	100127	101498
2 500	74433	75877	77997	76998	77738	75302	80967	83108	82170
1 250	62296	62993	63385	64393	65431	64192	66197	67426	66831
0	50267	50741	51935	50202	53223	47579	32014	29152	31287

The mean of the 9 values fluorescence intensity is done for each number of cells is done so the graph showed in *figure 4.11* can be done.

Table D: Mean of plate reader results (table used to draw calibration curve).

Cells number	Mean fluorescence intensity [a.u.]
80 000	818044,222
40 000	438512,889
20 000	215885,667
10 000	142822,222
5 000	100025,556
2 500	78287,7778
1 250	64793,7778
0	44044,4444