UNIVERSITÀ DEGLI STUDI DI PADOVA
DIPARTIMENTO DI INGEGNERIA INDUSTRIALE
CORSO DI LAUREA MAGISTRALE IN INGEGNERIA CHIMICA E DEI PROCESSI INDUSTRIALI

Tesi di Laurea Magistrale in
Ingegneria Chimica e dei Processi Industriali

DEVELOPMENT OF AN EXTRACELLULAR MATRIX HYDROGEL FOR INTESTINE TISSUE ENGINEERING

Relatore: Prof. Nicola Elvassore

Laureanda: FRANCESCA ONOFRI

ANNO ACCADEMICO 2015 – 2016

Abstract

In this work has been deepen the study of a hydrogel derived from decellularized piglet intestine thereby having the advantage of possessing extracellular matrix (ECM) information of native tissue that would be beneficial for many application in tissue engineering. These include the 3D-culture of organoids for the repopulation of decellularized scaffolds, hybrid engineered intestinal replacement or delivery of intestinal cells to restore function.
Two different methods for decellularization were investigated which were Detergent Enzymatic Treatment (DET) and SDS (sodium dodecyl sulphate) ionic solvent. A gelation protocol was developed, which involved freeze-drying, milling and digestion followed by neutralization to a physiological pH and temperature. Whole intestine and mucosa alone were compared examining gelation and rheological properties, and ECM composition. Finally the gel was investigated in order to determine its citocompatibility and for its potential to support intestinal organoids in 3D-culture conditions.

Index

Introduction

Tissue engineering (TE) aims to fully restore and repair damaged or degenerated tissues and organs using a combination of cells, bioactive molecules, and scaffolds. The importance of gaining success in this field is tragically clear. In the US for example, 77 people receive transplants each day but approximately another 20 die daily on the waiting list. There are currently over 98 000 patients awaiting transplants in the US, with a three year average waiting time for a transplant.[1] The lack of donors for organs is only one of the many problems associated with current therapeutic options, which include lifelong immunosuppression and the difficulties to find the organs of the right dimension.[2]

Gastrointestinal pathologies, injuries, and congenital defects can lead to intestinal failure and this affect millions of individuals each year. The most common cause of intestinal failure is short bowel syndrome where at least half or more of the small intestine has been removed. Short bowel syndrome is typically a postsurgical condition for treatment of conditions such as trauma or necrotizing enterocolitis. Intestinal failure may also be caused by functional disorders such as Crohn's disease, a digestive disorder, or chronic idiopathic intestinal pseudo-obstruction syndrome. Although there are several treatment options for these individuals, such as total parenteral nutrition or intestinal transplant, no ideal solution exists. The restoration or replacement of the gastrointestinal tissue, therefore, represents a large unsatisfied clinical need. To this end, this work focuses on the development of a naturally derived hydrogel that could represent a valuable tool in the treatment of intestinal failure

Although considerable progress has already been made in the development of organ replacements, there is still far to go. Many different types of hydrogels with varying chemical and physical properties have been developed and explored over the last several decades. These have ranged from completely synthetic polymers like PEG (poly(ethylene glycol)) to natural extracellular matrix (ECM) derived hydrogel like collagen or Matrigel. They each have their own associated advantages and disadvantages, making them suitable for different types of applications. A hydrogel that mimics the intricate biological identification and signalling functions of the intestine extracellular matrix for application in tissue engineering, repair and regeneration would be a very valuable tool for a variety of applications. These could hypothetically include cell delivery of autologous cells to restore function of damaged tissues, the 3D-culture and expansion of organoid stem cells for the repopulation of decellularized scaffolds or as the ECM component of a hybrid engineered intestinal replacement.

Therefore, the aim of this study was to prepare, characterize and test the in vitro citocompatibility of an ECM hydrogel derived from the decellularization of piglet intestine tissue. It is considered that different biomaterials obtained from extracellular matrix (ECM) have already been employed to replace different tissues of the body in preclinical and clinical studies[3].

The first chapter introduces the field of tissue engineering, discusses the relevant literature and describes the aim of this work. The second chapter describes the materials and the techniques used for the study. This is broken into three sections; i) the decellularization process and its characterisation, ii) the preparation of the gel and the gel characterization including rheological and turbidimetric analysis and iii) cytocompatibility testing and organoids culture. The third chapter outlines the results, and the fourth and final chapter discusses the results and conclusions with mention of suggested future developments.

Chapter 1

Introduction and state of the art

This chapter provides an introduction into the field of tissue engineering as well as a brief overview of the various potential applications of an extracellular matrix hydrogel.

1.1 Tissue Engineering

Tissue engineering (TE) is an interdisciplinary field which aims to restore, maintain or improve biological tissue function using the principles of engineering and life sciences, combining the use of scaffolds, cells, and biologically active molecules.[4] The goal of tissue engineering is to surpass the limitation of conventional treatments such as organ transplantation as these are limited by a lack of suitable donors, the necessity of lifelong immunosuppression[2] and the difficulties of finding organs of the right dimension specifically for paediatric patients.[5] The creation of 'artificial' organs and tissue substitutes using the patient's own cells has the potential to be a viable treatment option for damaged organs and tissues. This could potentially eliminate the need for immunosuppressive therapies and could rectify the shortage donor problem, thus leading to a cost-effective improvement in the long term.[5]

Tissue engineering typically involves three base concepts; i) selected and isolated cells (for example progenitor or stem cells) ii) biomaterial scaffolds which may be natural or synthetic or a hybrid of the two iii) signalling molecules such as growth factors or proteins[6]
There are many approaches for developing replacement tissues and organs. Many of these approaches involve isolating the patient's cells, which are tissue specific, from a small biopsy, expanding them in culture and seeding them onto 3D scaffolds, which often aim to mimic the natural ECM. The main function of the scaffold is to support the seeded cells and deliver them to the desired site, to promote cell adhesion, and to mechanically maintain the structure of the engineered tissue.
A commonly used approach to create organ replacements is to decellularize the native tissue. This technique involves removing any cellular material present in the tissue while also conserving the extracellular matrix as much as possible. There are presents many protocols in the literature and they often include a series of detergents and/or enzymatic treatments. This

procedure has been performed successfully for many organs such as trachea, small intestine, liver, kidney, hearth and lungs, and has the advantage to create scaffolds which are already bioactive and tissue specific, moreover the structure of the organ is often preserved.[7 8 9]

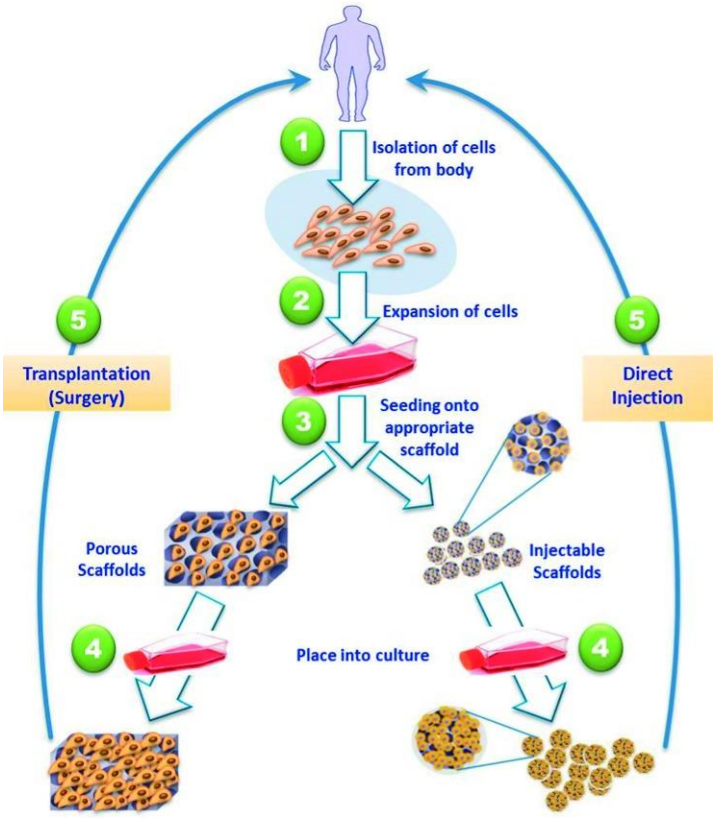

Figure 1.1. *Schematic illustration of the most common tissue engineering approaches*[6]

1.2 Extracellular matrix

The Extracellular matrix contains secreted products of the resident cells of each tissue and organ. The ECM was initially considered an inert scaffold whose main role is to provide mechanical strength to the tissue, however, today it is accepted as a three-dimensional structure that facilitates the survival of cells by playing an active role in regulating biologic processes, providing physical protection and signals, directing and facilitating cell behaviour such as proliferation, orientation, gene expression, migration and differentiation.[10] It is important that the functional components of the ECM contribute to provide an ideal microenvironment specific to each tissue and organ. For this reason, ECM composition can differ significantly between tissues types. For example the solid calcified structure of the bone differs greatly from the soft and transparent matrix of the cornea.[5]

Fundamentally, the ECM is composed of water, polysaccharides and proteins but each tissue has a specific composition and topology that is the result of the dynamic interactions that occur during tissue development between different cellular components such as fibroblasts, epithelial cells, adipocyte, and the protein environment.[11]

The ECM is responsible for the biochemical and mechanical properties of each organ, including its tensile and compressive strength and elasticity. Moreover it has a buffering action that maintains extracellular homeostasis and water retention. The ECM binds also grow factors (GFs) that interact with cell-surface receptors regulating gene transcriptions and eliciting signal transduction.

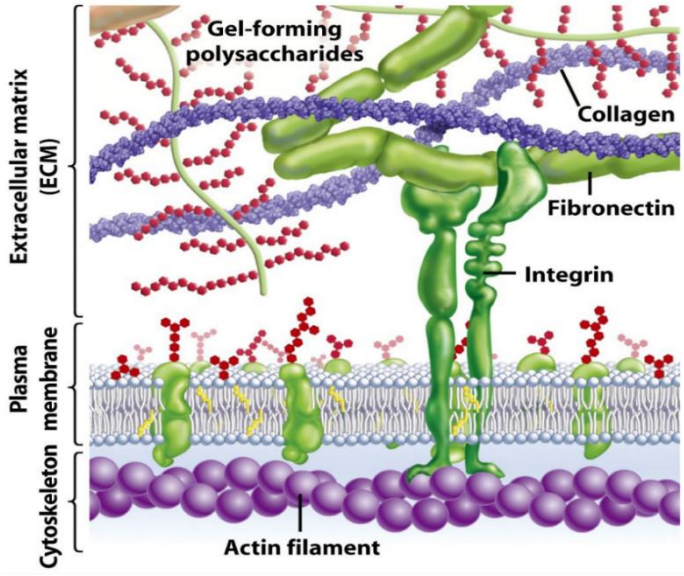

Figure 1.2. *Principal ECM components.*[55]

Two main classes of macromolecules are present: proteoglycans (PGs) and fibrous proteins [10]. PGs make up the majority of the interstitial space in the ECM and appear has a hydrated gel. Collagen, elastin fibronectin and laminin represent the main fibrous components of the ECM, whose main role is to resist tensile stress.

Collagen makes up 30% of the total protein mass of a multicellular animal and so it represents the most abundant component in the ECM. It is responsible for the tensile strength, regulation of cell adhesion and direction of tissue development.[12]

Collagen fibres are very heterogeneous, but usually in each different tissue there is one type that is predominant, for example in intestine collagen type I is predominant. Collagen is a right-handed triple helix structure that can assemble into supramolecules complexes as fibrils and networks depending on the type of collagen. The arrangements of collagen molecules is usually stabilized by covalent cross-links formed by lysine and hydroxylysine aldehydes. [13]
These molecules are largely insoluble except after protolithic release (e.g. pepsin). Currently, 28 different type of collagen have been identified, of these collagen type I is the most abundant in the human body as it is present in skin, tendons and connective tissue.[13]

Elastin is another important fibrous component of the ECM. It permits tissue recoil after repeated stretch, allowing the tissue to recover to its initial shape, and thus confers elasticity to the tissue. The secreted precursor of elastin are Tropoelastin, which assemble into mobile hydrophobic regions via enzymatic cross-linking of their lysine residues.[11]

Fibronectin (FN) is an ECM glycoproteine that assembles into a fibbrillar matrix and is responsible for directing the organisation of the interstitial ECM.
FN forms linear and branched meshwork around cells and connects neighbouring cells, contributing to the mediation of cell attachment and function.[14]
It is a dimeric protein composed of two almost identical subunits covalently bound together by a pair of disulphide bonds. Cellular traction force can stretch FN several times over its resting time. FN has a wide variety of functional activities other than binding to cell surfaces through integrins. It binds to a number of biologically important molecules that include heparin, collagen/gelatin, and fibrin but also plays an important role in cell migration, growth and differentiation.

Laminin is one of the major components of the basal lamina, a layer of the basement membrane that is secreted by epithelial cells. It creates a network that is the foundation for many cells and is associated with type IV collagen networks via entactin, fibronectin, and perlecan. It contributes to cell attachment and differentiation, cell shape and movement and promotes tissue survival through receptors interactions which are derive from the fact that laminin binds to cell membranes through integrin receptors and other plasma membrane molecules.[15] Laminins are present as an α , β, and γ chain form (with five, three, and three different variants identified respectively) and could assume 15 different combinations. They assume a cruciform structure with a longer arm that is a coil-coiled region of the three shorter arms (the extremities of α, β, and γ chains).[16]

Protoglycans play an important role in controlling proliferation, differentiation and motion. It is now clear that stimulates the proliferation of fibroblasts and chondrocytes, through the presence in the molecule of EGF-like motifs.[17]
Proteoglycans are formed of a protein core and glycosaminoglycans (GAGs) chains fixed to it. GAGs are negatively charged polysaccharides formed of disaccharide units that might be nonsulphated, like hyaluronic acid which does not form proteoglycan, or sulphated like chondroitin, dermatan, keratan, and heparan sulfates. GAGs due to their polar nature attract cations that in turn will attract water by osmosis. Thus GAGs form a highly hydrated network that gives this gel-like structure to the ECM and allows it to withstand compressive stresses. They also have the role of growth factor reservoir, in fact grow factors, which are molecules that modulate cellular activity, are stabilized and protected from proteolytic degradation by their interaction with GAGs, which modulate a sustained released of these.

Until now it has been difficult to replicate the complexity of ECM in its entirety using biomaterials [18] or mimicking its composition using purified ECM component.[19]
Also considering that synthetic biomaterials could potentially mimic the structural environment they could leads to cytotoxic degradation by-products where implanted with a consequent inflammation area.[20] One alternative to synthetic biomaterials is to obtain the native ECM from the tissue by removing the cell content using different types of treatment, which has already be done for some tissue such as bladder, heart, liver, and lung. [21]

1.3 Decellularization Methods

In literature are reported different techniques which are used for the decellularization of organs and tissues. Usually there is a distinction between fiscal, chemical, and enzymatic methods. Physical treatments includes agitation, sonication, pressure, frosting and defrosting; These methods destroys cellular membrane allowing the release of cellular contents and making easier their removal from the ECM.[22] Physical treatments are not sufficient alone for the obtainment of a complete decellularization and so they are combined with chemicals treatments. The combination of different approaches is usually adopted for the maximisation of the decellularization effects. Enzymatic treatment, as for example the use of trypsin, and chemicals treatments, as for example the use of ionic solutions and detergents, destroy the cellular membrane and the bonds responsible for the intra and extra-cellular connections.[23] The most effective decellularization protocols present a combination of physical chemical and enzymatic treatment:

- 1° step: lysis of the cellular membrane through a physical approach (agitation, pressure, freezing, and de-freezing) or through ionic solutions;

- 2° step: solubilisation of cytoplasm and nuclear components thanks to chemical detergents.
- 3° step: separation of cellular components from the ECM through enzymatic tools;

These steps can occur, for example, with mechanical agitation in order to increase process efficacy. After the decellularization all the chemical residue must be removed to avoid any adverse response from the host tissue.[22]

1.3.1. Physical methods

Physical methods that can be used to facilitate the decellularization of tissues include freezing and de-freezing, application of pressure, mechanical agitation and electrophoresis. [24] A rapid freezing of a tissue involve the formation of ice crystals inside the cells which cause cellular lysis after the membrane breaking. Freezing and de-freezing processes effectively destroy cells of organs and tissues but intracellular contents and membrane residues stay inside if not removed through subsequent processes.
It has been shown that one cycle of freezing and de-freezing is able to lower the immune adverse response, and repeated cycles tend to minimize adverse immune response without involving a significant lost in the membrane protein from the tissue. This process has minimal consequences on ECM ultrastructure and mechanical properties and on tissue mechanical properties maintenance.[22] Hydrostatic pressure for a relative low time results to be more efficient than detergents and enzymes in cellular removing.
Electroporation is the application of electrical pulsations of microseconds through the tissue and tend to cause the formation of micro pores in the cellular membrane due to the instability of electrical potential. These micro-pores are responsible for homeostasis and cells death.
Limitations of this technique is the dimension of the probe, which are small and so limit the dimension of the tissue that can be decellularized. Moreover, the decellularization process must be carried on in vivo since it is mediate from the immune system.
It is always better to associate a perfusion technique to electroporation in order to eliminate all the residue left.
Mechanical agitation and sonication are usually used together with chemical treatments for the elimination of cellular debris after cells lysis. Mechanical agitation is usually obtained with a shaker or a magnetic stirrer. In both cases velocity, reagent quantities and duration depend on composition, volume and density of tissue.

1.3.2 Chemical methods

Since all chemical reagents for the cellular removal alter ECM composition one of the objective of decellularization is the minimization of these adverse effects.
Acids and bases provoke or catalyse the hydrolytic degradation of biomolecules, solubilize the cells cytoplasmic component, remove nucleic acids such as DNA and RNA. [22]
For example acetic acid, paracetic acid, ammonium hydroxide can actually destroy cellular membrane and intracellular molecules, but at the same time they dissociate important molecules such as GAG from tissues rich in Collagen.
Hypertonic saline solutions dissociate DNA from proteins, while hypotonic solutions can cause cellular lysis for osmotic effect with minimal consequences on matrix architecture.
In order to increase osmotic effect it is possible to submit the tissue to different cycles of alternate hypotonic and hypertonic solutions; these solutions also comport a rinse of the cellular residues from the tissue.[22]
Non ionic, ionic and Zwitterionic detergents solubilize cellular membrane and dissociate DNA from proteins, are very efficient on removing cellular residues from the decellularized tissue. Non ionic detergents destroy the lipid-lipid and lipid-protein interaction, leaving the protein-protein interaction intact. Ionic detergents, instead, are capable of destroying also protein-protein bond.
Triton X-100 is the most commonly adopted ionic detergent for the thicker tissues were enzymatic and osmotic methods are not sufficient.
Sodium dodecyl sulphate (SDS), sodium deoxycholate (SDC) and triton X-200 are the most commonly adopted ionic detergents. SDS satisfactory remove cellular components from the tissue but imply the destruction of the native structure of the tissue, with a GAGs concentration diminishing and lost in collagen integrity, also if it its removal from the tissue has not been demonstrated.
SDC has positive effects on cellular and residues removal but is more aggressive in the removal of the native architecture of the tissue compared to SDS.
Zwitterionic detergents presents some properties which are in between ionic and non-ionic detergents, but have the tendency to provoke protein denaturation. Tri(n-butyl)phosphate (TBP) is an organic solvent which is commonly adopted for the inactivation of virus presents in the blood. Only recently it has been used as a decellularization agent. It presents good decellularization capacity without bringing damages on mechanical properties of ECM, for examples without disrupting collagen fibres. For this reason it appears as a promising decellularization agent.[24]
Finally chelant agents, such as EDTA and EGTA, are molecules that build molecular ring unit which bind and isolate a central metallic ion. Are used to remove cells from protein substrate. This occur thanks to the sequestration of bivalent cations such as Ca^{2+} e Mg^{2+}, by the molecular

ring. These cations are necessary for the adhesion of cells to collagen and fibronectin. Binding these cations, chelants gents facilitate the removal of the cellular materials from the tissue. Are usually combined with other substances since alone they are ineffective.[24] EDTA is used together with trypsin. When operating with chemical methods it is of primary importance to ensure the complete removal of any chemical residues from the ECM after the decellularization. These agents could result toxic for the cells in the host tissue after the scaffold implantation.[22]

1.3.3 Enzymatic Methods

Enzymatic method involve the use of enzyme of digestion or nuclease. Trypsin is one of the most adopted protolithic enzyme in the decellularization protocols. It is responsible for the breakage of the peptide bonds at the carboxyl ends of Arg and Lys, and its active at 37 °C and pH= 8. ECM components usually have a limitate resistance to trypsin, so it has to be used with caution.[22]

Compared with detergents it is more destructive toward elastin and collagen, but usually is less destructive toward GAGs content. Its action is slow and a complete decellularization with the use of only trypsin is almost impossible since it will require a too long incubation time.

For this reason trypsin is used together with other agents. It destroy the tissue ultrastructure and facilitate the permeation of the others decellularization agents.[24]

Nuclease catalyse hydrolysis of DNA and RNA bonds causing their degradation. Also in this case it is important the complete removal of the enzyme after the decellularization process in order to avoid adverse immune response in the host tissue.[24]

1.3.4 Decellularization Protocols

The efficacy of a method or of a protocol of decellularization is dependent on the tissue nature. Different studies have shown that origin, composition and density are factors which affect the success of the decellularization. This is why it is important to study a different protocol for each organ and tissue.[24]

Gauss et al. [25] have observed how a protocol based on the use of only trypsin is ineffective on the decellularization of an aortic mouse valve. While the same treatment is effective for the pulmonary porcine valve as demonstrated by Schenke-Layland et al.[26]

More over a combination of physical chemical and enzymatic treatment improve the efficacy of decellularization protocols.

In fig. 1.3 are reported some of the different decellularization protocols in which different methods are combined together. It is observable how protocols are very different between different types of tissue.

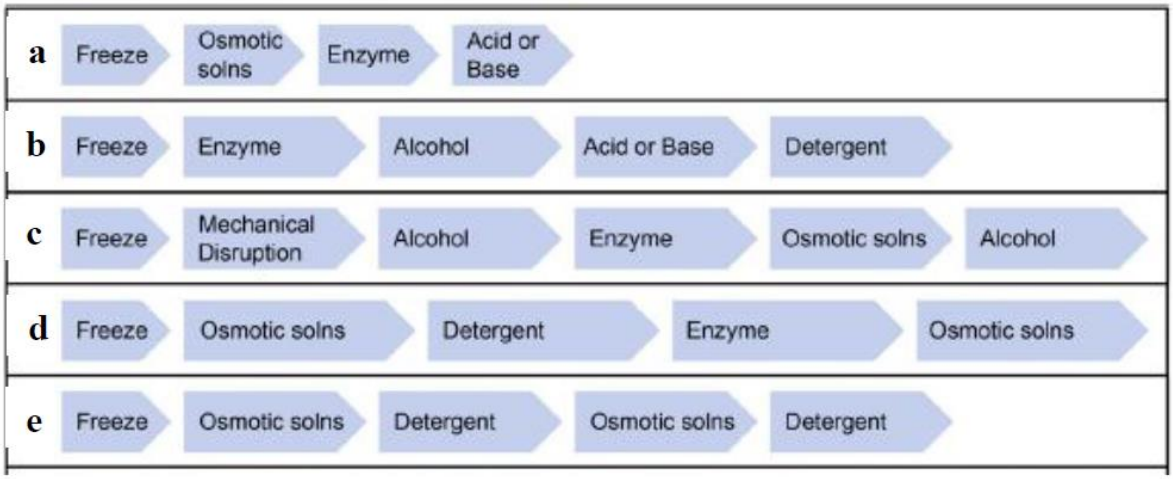

Figura 1.3 *Examples of different protocols of decellularization for a) thin layers as pericardium, b) thicker tissues as derma c) amorphous tissues with presence of fat as adipose tissue d) simple organs such as trachea e) and complete organs liver.*

1.4 Hydrogel

Regardless of the widespread use of materials in medicine, many biomaterials aren't good candidates to interface with biological systems and have not been engineered for optimized performance. Therefore, there is a rising demand to develop optimized materials to solve such problems in different fields as medicine and biology. The cross-linked form of hydrophilic polymers, known as hydrogels, are a class of biomaterials that have demonstrated great potential for this fields.[27]

Hydrogels are three-dimensional networks made of hydrophilic polymers which are crosslinked via covalent bonds or via physical attraction, either intramolecular or intermolecular. They have the ability of absorb and retain a huge amount of water (often more than 90%) and swell without dissolving. Hydrogel are soft and rubbery in the swollen state which make them look like living tissue, for this reason they are used nowadays for many application in tissue engineering. The principal use is as scaffolds that mimic the ECM environment and for 3D cell cultures or as a tool to encapsulate and deliver cells.[6]
In cell transplantation hydrogels have the beneficial ability of create an immunoisoleted environment and at the same time allowing the diffusion of nutrients oxygen and metabolic product.
As scaffold hydrogels have the ability of mimic the mechanical characteristic of natural tissue and they can be used directly after their preparation or after the creation of a new tissue, their use is to ensure bulk and mechanical structure where cell can be incorporated or suspended in the 3D structure.

There are many different ways in which hydrogel can be classified. First of all they can be physical networks that are the result either of polymer chain entanglements or physical interaction such as ionic interaction, hydrogen bonds or hydrophobic interactions or chemical cross-linked by covalent bonds. These interactions are controlled by the physical condition and can be desegregated by changing ionic strength, pH, temperature, a stress application, or addition of some solutes that compete with the polymeric ligand for the site of affinity on the protein.
Physical hydrogels are not homogeneous due to different factors that can create inhomogeneities such as clusters of molecular entanglements, or hydrophobically or ionically associated domains.[28]
Hydrogels are called 'chemical' gels when they are covalently-crosslinked networks and they do present permanent properties. It is also possible to classified hydrogel according to their origin which can be natural or synthetic.

Synthetic hydrogels such as poly(ethylene glycol) (PEG), polyglycolic acid (PGA), polylactic acid (PLA) have the advantages to offer a high degree of the control of the properties, in fact they can be synthetize with molecular scale control of the structure such as crosslinking density, or of the properties such as mechanical strength and biodegradation.[29]
In the other hand natural derived hydrogel are considered to demonstrate more adequate biocompatibility, while synthetic hydrogel may elicit significant inflammatory response that can affect the immune response toward the transplanted cells. [5]
Moreover synthetic hydrogels [30] lack the endogenous factors such as bioactive molecules that promote cell behaviour and act mainly as a template to permit cell function such as the adhesion, growth, proliferation, differentiation, and ECM secretion of embedded chondrocytes. These polymer scaffolds can eventually degrade and be absorbed by the body through the metabolism process.
Thus designing a synthetic hydrogel is very useful if a small set of specific properties is desired, but on the other hand reproducing the whole complexity of the extracellular matrix itself in a synthetic hydrogel is a colossal if not impossible task.

Natural hydrogels are typically formed of proteins and ECM components such as collagen, fibrin, hyaluronic acid, or Matrigel, or they can also originate from other biological sources such as plants or animals, for instance chitosan (from crustacean), alginate (from algae) or silk fibrils [31]. These gels have the advantage of already being biocompatible and bioactive[32].
Natural derived hydrogels have the disadvantages of not being completely known in term of composition and so difficult to be reproducible presenting a large batch to batch variation.

Furthermore their physicochemical properties are also difficult to tune, such as their degradation rate that can be too fast, they have poor mechanical properties.

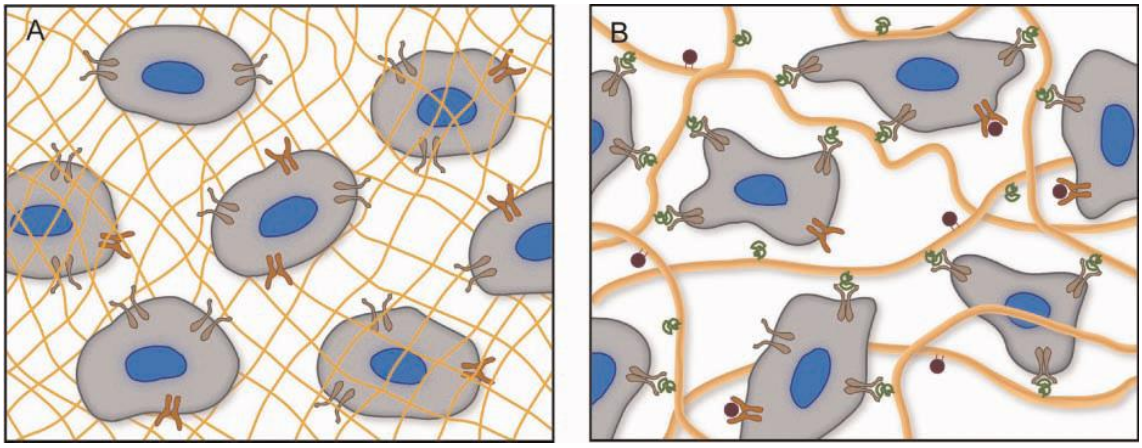

Figure 1.4. *Permissive hydrogels (A) composed of synthetic polymers (yellow mesh) provide a 3D environment for culturing cells; however, they fail to activate integrins (brown) and other surface receptors (orange). The synthetic environment simply permits viability as cells remodel their surrounding microenvironment. On the other hand, promoting hydrogels (B) formed from naturally derived polymers present a myriad of integrin-binding sites (green) and growth factors (red) coordinated to the ECM (yellow fiber)*[56]

They have the ability to promote cellular functions thanks to the presence of endogenous factors which can be advantageous for the viability, proliferation, and development of many cell types. However, such scaffolds are complex and often ill-defined, making it difficult to determine exactly which signals are promoting cellular function [31]

Collagen is one of the most common used hydrogel and has the advantage to meets many of the biological design parameters because of its composition which is recognized by cells and is naturally biodegradable by enzymes secreted from the cells such as collagenase.
Collagen has been used as a tissue culture scaffold or artificial skin, and for the reconstruction of liver, skin, blood vessel, and small intestine.[33]

Matrigel is another commonly used as a 3D natural hydrogel commonly used both in vitro and in vivo. It is a complex gelatinous mixture of basement membrane ECM proteins extracted from Englebreth-Holm-Swarm tumor in mice. It is mainly formed of laminin, collagen type IV and also contains several growth factors which can promote epithelial cell grow.[21].
However its exact composition is not well known, which can cause some variability between experimental results. Matrigel has many different applications; An important one is to aid in human embryonic stem cell culture, as it has the ability to maintain self-renewal and pluripotency [34]. It can also be used for the organoid culture *in vitro*.

1.4 Intestine

The small intestine is that part of the gastrointestinal tract which is positioned between the stomach and the colon. It can be divided in 3 different regions: the duodenum, the jejunum, and the ileum (Figure 1.5.). Blood is supplied to intestine mainly by a single blood vessel, the superior mesenteric artery, and exits it through the superior mesenteric vein.

The intestinal wall is composed of 4 distinct layers, which are the mucosa, the submucosa, the muscularis externa, and the serosa, from the lumen to the outside layer.

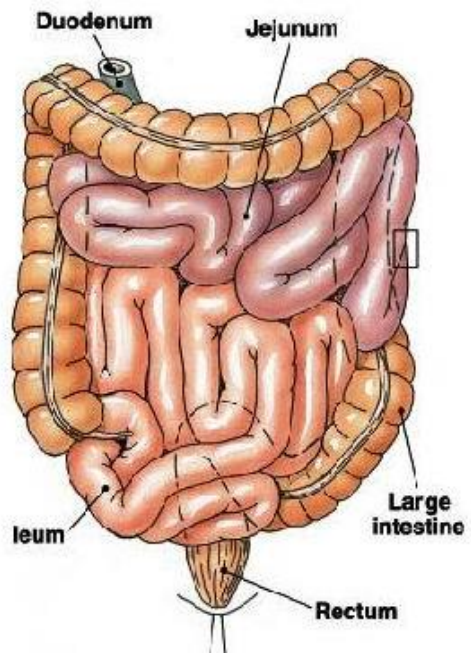

Figure 1.5. *Regions of the Small Intestine*

The mucosa is composed of a layer of epithelial cells organised in villi and crypts, and some connective tissue underlined by a thin layer of smooth muscle. The nutrients absorption is due to the presence of a lot of capillaries passing through the epithelial cell layer, and the immune role is provided by the presence of as well as lymphatic nodules. The submucosa is mainly composed of connective tissue. The muscularis externa is composed of two different smooth muscular layer, a circular one and a longitudinal one. By the production of cyclic waves of radial contractions (peristaltic) the processed food is pushed by the muscular layer through the intestine.

The rest of the intestine is finally protected by the external membrane called serosa which is formed of two layers of epithelial cells and has the ability of producing a lubricating fluid that minimizes the friction between the intestine and the rest of the organs and/or tissues.

The inner epithelial layer of the small intestine is a multitasking tissue which has the role to ensure an efficient digestion and also absorption of the nutrients, while maintaining an effective barrier against potentially lethal microorganism.[35] That is why intestine structures has developed a way to maximise its surface area by forming a large circular fold called the picae circulares, and additionally the mucosa is covered by loads of protuberances called villi, and on the micro-level mucosal cells are covered by small microvilli structures on the plasma membrane. The persistent aggression from the luminan environment induce a high rate of cell death, with up to 1011 epithelial cells being lost every day in humans. The need of daily self-renewal is driven by a population of adult stem cells that generate new functional epithelia. [35]

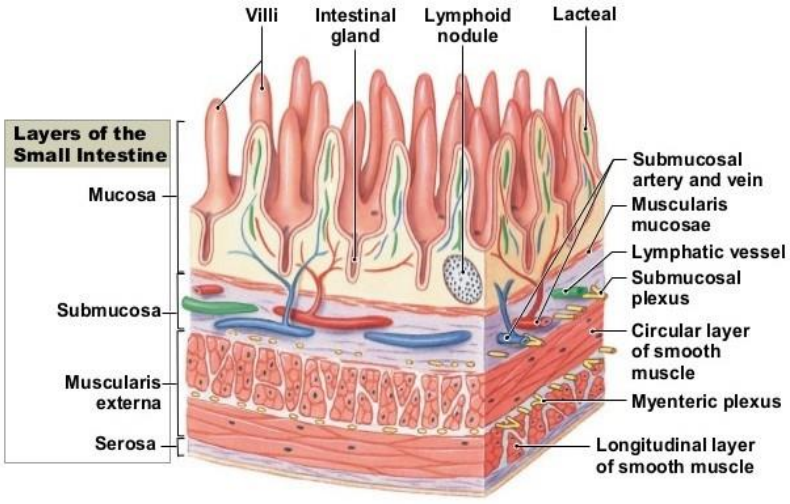

Figure 1.6. *Organisation of the intestine wall*

Intestinal failure is the endpoint of multiple disease processes affecting the intestine, all of which are characterised by an inability to maintain adequate nutrition (protein energy, fluid, electrolyte and micronutrient balance).
It can result from intestinal obstruction, abnormal motility of the gut, major surgical resection (as a result of ischaemic injury to the gut), congenital defects (gastroschisis, malrotation, intestinal atresia), diseases of prematurity (necrotising enterocolitis) or disease associated loss of absorption (such as in Crohn's disease).
Loss in intestinal segments due to congenital deses or multiple surgical resections due to inflammation or cancer result in the short bowel syndrome, which cause malabsorption, dehydration, electrolyte abnormalities and failure to thrive.[36]

The Current therapeutic options for SBS are limited and include bowel lengthening procedures or total parenteral nutrition (TPN) which is not curative. This therapy carries a high morbidity, uses extensive healthcare resources, and diminishes the patient's quality of life[36].

The promising treatment would be small bowel transplantation, however this solution require a donor of appropriate tissue and long term immunosuppression and carry significant risks as morbidity and mortality.[37]

A tissue engineering approach could offer a potentially advantageous solution for creating a functional intestinal absorptive area avoiding the complication of currently therapeutic options, since engineering a new intestine *in vitro* using autologous cells of the patient would solve the issues that exist with allografts. The smallest mucosal unit which can be transplanted are intestinal organoids. These are multicellular crypte-like structure s of 20-40 cells which can be isolated from mucosa crypts and that contain the intestine stem cells that comprise the stem cell nich.[21]

1.5 Intestinal Organoids

Organoids are structures which resemble whole organs generated from stem cells through the development of three dimensional culture systems. Organoids are derived from pluripotent stem cells or isolated organ progenitors that differentiate to shape an organ-like tissue exhibiting multiple cell types that self-organize to form the cellular organization of the organ itself.
The therapeutic promise of organoids is that they could potentially model developmental diseases, degenerative conditions, and cancer. Furthermore, organoids that model disease can be used as an alternative system for drug testing that may not only better recapitulate effects in human patients but could also reduce tests on animal. Finally tissues derived in vitro could be generated from patient cells to provide alternative organ replacement strategies. Unlike current organ transplant treatments, such autologous tissues would not suffer from issues of immunocompetency and rejection.[38]

It has been recently highlighted that in a three-dimensional cultures Lgr5 (marker of stem cell in multiple adult organs of mice and humans) stem cells can grow into ever-expanding epithelial organoids that retain their original organ identity. Single stem cells derived from the patient intestine can be cultured in order to build epithelial structures that maintain hallmarks of the in vivo epithelium. [39]
Intestinal stem cells are positioned near the intestinal crypt bottom. Their daughter cells divide rapidly and occupy the remaining part of the crypts moving onto the border of the villi. There they differentiate, absorb nutrients, and eventually die at the villus end.
The differentiated cell take in number different types which are absorptive enterocytes, different secretory cells (Paneth cells, goblet cells, enteroendocrine cells, and tuft cells), and the Peyer's patches31 M cells. Somatic cells have a proliferative potential which is limited to the fact that genetic transformation may be induced from a long term culture. That is why it has always been

very difficult to establish a long time culture from primary adult tissue. Recently Sato et all. have established an in vitro system for the culture of three-dimensional intestinal epithelial organoids from a single Lgr5-CBC cell for a timing greater than 1.5 years.
These Lgr5-CBC are crypt base columnar (CBC) cells at the crypt bottom and they are the dividing Lgr5+ stem cells which they divide every 24 hours generating 16 to 32 differentiated epithelial cells per day.

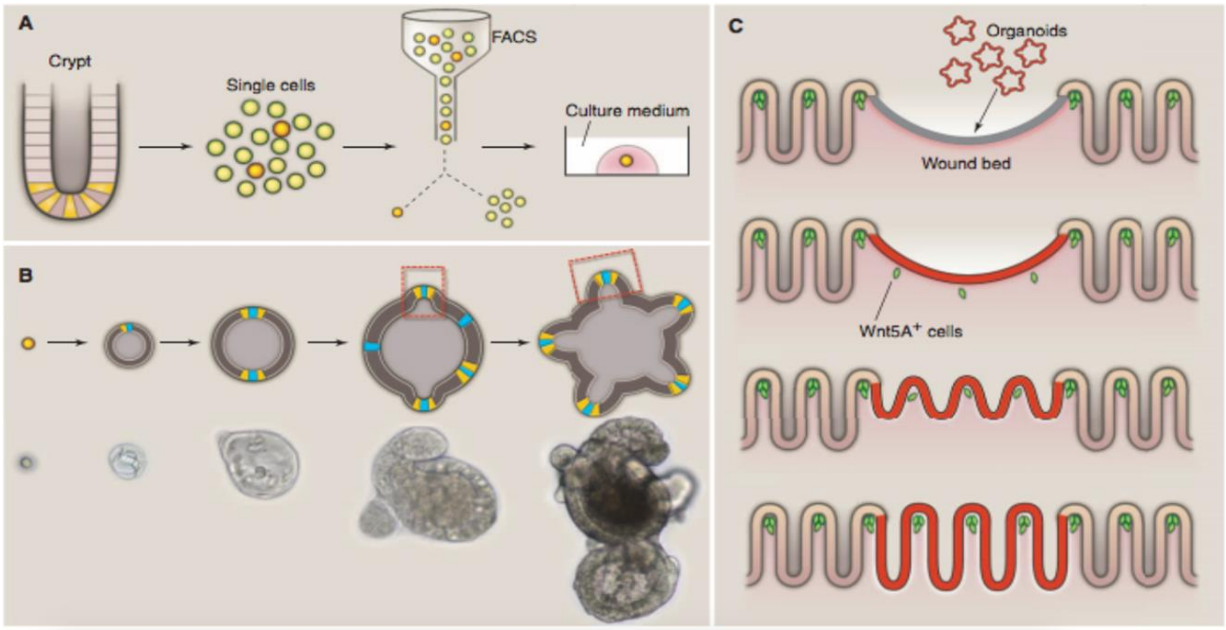

Figure 1.7. *Formation of intestine organoids from single cell grown on Matrigel: (A) Single cells are obtained from crypts, then FACS sorted, Lgr5 positive CBC cells are isolated and then embedded in Matrigel surrounded by culture medium made of EGF, Noggin, and R-spondin. (B) Formation of the organoids from a single cell, Lgr5 positive CBC cells shown in yellow and paneth cells in blue.*[39]

Single crypts can be isolated from intestine biopsy by EDTA-based Ca^{2+}/Mg^{2+} chelation. These can then grow into 3D organoids if placed in Matrigel and a mixture of R-spondin, EGF, and Noggin factor cocktail. In vitro organoids appear as cysts with a central lumen bounded by highly polarized villus epithelium and were reseeded into the colons of different mice with mucosal lesions. The epithelium derived from these mini-gut was indiscernible from surrounding original epithelium. An epithelial mini-gut grows logarithmically and expands 1000-fold within a month.

1.6 Aim of the project

Organ morphogenesis in vitro has been hindered by the lack of appropriate culture systems that would allow the cell-cell interactions needed for organ formation. In order to optimize the conditions that augment neomucosal growth and architecture by organoids propagation in vitro

the aim of this project is to develop a natural derived hydrogel obtained from piglet intestinal extracellular matrix. Since it has been proved that organoids can be potentially used to regenerate the intestinal epithelium it is necessary to find a validate substitute to Matrigel for the in vitro culture. Matrigel (BD Biosciences, San Jose, CA), a 3D laminin and collagen-rich matrix that mimics the basal lamina, which nowadays represent the main solution in organoids culture, can't be used in tissue engineering in humans. Because of its derivation from mouse sarcoma Matrigel will provoke immune responses if used in other species.

The steps that have been followed are first of all the optimisation of an established decellularization protocol for this new tissue and technique, in order to obtain a decellularized ECM. The potential benefits of using ECM hydrogel derived from homologous source tissue include the retention of tissue specific cell phenotypes, enhancing tissue-specif differentiation and promoting proliferation of progenitor cells [19] [40], which could partially recapitulate the complexity of mammalian organogenesis in vitro.

Secondly it has been studied the formation of a gel, with the characterization of the gelation kinetic, rheological and viscoelastic properties. Lastly were performed test on citocompatibility of the product first on fibroblast cells and second on intestinal organoids.

Chapter 2

Materials and Methods

In this chapter the materials and methods used for the production of the Hydrogel are presented together with the method utilized for its characterisation.

2.1 Tissue harvesting

The tissue used for this experiment is piglet small intestine, which was harvested from a Synthetic Pietrain breed. All animals were newborn females less than one day old. They were sacrificed by blunt trauma following industry and veterinary protocols. The tissue was collected either at the Royal Free Hospital and then brought to the Institute of Child Health (ICH), or directly retrieved at ICH. Two types of tissues were used, the whole intestine and the submucosa & mucosa layer of the intestine (from now one called (sub)mucosa). The muscle was removed using a procedure developed which consisted of gentle delamination using surgical techniques performed *in situ*. Removal of the (sub)mucosa layer on recently sacrificed piglets (>12 hours) has shown to be easier compared to the separation after this. After retrieval, tissues were carefully cleaned by removing any content and then stored in at -80 ° C until future use.

2.2 Tissue decellularization

The most effective agents for decellularization of each tissue and organ depend upon a number of different factors, including the tissue's cellularity (e.g. liver vs. tendon), density (e.g. dermis vs. adipose tissue), lipid content (e.g. brain vs. urinary bladder), and thickness (e.g. dermis vs. pericardium).[22] Every method and agent for cell removal will alter ECM composition and cause a different degree of ultrastructure disruption which is why it is very important to characterise your tissue post-decellularisation when using a new protocol. Different protocols exist and the two most-commonly used were investigated here. These are considered to be a good balance between the desired results of decellularization, DNA content, and minimization of the undesirable effects, which is the ECM disruption.[7]

Both DET (detergent enzymatic treatment) and SDS (sodium dodecyl sulphate) have been shown to be very efficient in removing nuclear remnants and cytoplasmic proteins from dense

tissues but tends to disrupt native ultrastructure, removes GAG and growth factors and damages collagen.[41] Before the use of each protocols tissue was freezed at -80°C for the disruption of cell membrane by intracellular ice crystal. The DET protocol uses SDC (sodium deoxycholate from SIGMA® life science)and begins with a washing step in MilliQ water (highly purified deionized water) at 4°C over night, hypotonic solutions can readily cause cell lysis by simple osmotic effects with minimal changes in matrix molecules and architecture. The next step is 4 hour in a 4% weight solution of SDC for the solubilization of cell membranes and dissociation of DNA from proteins, these agents also disrupt and dissociate proteins in the ECM. A 30 minutes washing step in PBS (phosphate buffer saline from SIGMA® life science) follows, and then a 3 hours wash in a 1M NaCl ,22.5 mg DNase (Desoxyribonuclease I from bovine pencreas from SIGMA® life science) solution for the elimination of the remaining DNA content as previously described[8]. Enzymes can provide high specificity for removal of cell residues or undesirable ECM constituents. However, complete cell removal by enzymatic treatment alone is difficult and enzyme residues may impair recellularization or evoke an adverse immune response. Nucleases (e.g. DNAses and RNAses) cleave nucleic acid sequences and can therefore aid in removal of nucleotides after cell lysis in tissues.

The removal of ECM proteins and DNA by detergents depend on the amount of time the tissue is exposed to the detergents and enzymes. The speed of decellularisation will also depend on organ subunits, tissue type, and donor age. For this reason, in these experiments, both one and two cycles of the decellularisation protocol were performed on the tissue and the optimum cycle number was determined. [22] After the decellularization the tissues were washed in MilliQ water at 4 °C for three days in order to remove detergents and enzymes which are toxic to the cells. All the washes were performed by immersion while being subjected to agitation through a magnetic stirrer, that can lyse cells, but more commonly is used to facilitate chemical exposure and removal of cellular material. The tissue was cut into small pieces of 1 cm^2 to provide as much surface area for the decellularization as possible and avoid the formation of a knot.

The second method investigated involved immersing the tissue in SDS (sodium dodecyl sulphate) for 24 hours. The addition of a detergent such as SDS to a decellularization protocol can make the difference between complete and incomplete cell nuclei removal but has the associated drawback of ultrastructure disruption and growth factor elimination.[42] .The protocol involves washing the tissue in MilliQ water (highly purified deionized water) at 4°C over night. This causes cell lysis by simple osmotic effects with minimal changes in matrix molecules and architecture. The tissue is then placed in a 0.25% weight solution of SDS followed by a three day washing in MilliQ. This procedure was carried on using an immersion method at room temperature with continued agitation. Samples after both one and two days in SDS were investigated. [43]

Table 2.1. *Selected agents and techniques for decellularizing tissue*[42]

Agent/Technique	Mode of action	Effects on ECM
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock, disrupt DNA-protein interactions	Effectively lyses cells, but does not effectively remove cellular residues
Ionic detergents		
- Sodium dodecyl sulfate (SDS)	Solubilize cell and nucleic membranes, tend to denature proteins	Effectively removes nuclear remnants and cytoplasmic proteins from dense tissues, tends to disrupt ultrastructure, removes GAG and growth factors and damages collagen. Mixed results with efficacy dependent on tissue thickness, some disruption of ultrastructure and removal of GAG
- Sodium deoxycholate		
Biologic Agents Enzymes		
Nucleases	Catalyze the hydrolysis of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue, could invoke an immune response
Physical and Miscellaneous Agents		
Temperature (freezing and thawing)	Intracellular ice crystals disrupt cell membrane	Ice crystal formation can disrupt or fracture ECM
Techniques to Apply Agents		
Agitation	Can lyse cells, but more commonly used to facilitate chemical exposure and removal of cellular material	Aggressive agitation or sonication can disrupt ECM

SDS is very effective at removing of cellular components from tissue. Compared to other detergents, SDS yields more complete removal of nuclear remnants and cytoplasmic proteins, but at the same time it has been shown to disrupt the native tissue structure, and causes a decrease in the GAG concentration and a loss of collagen integrity[42]. Care must be taken to flush residual chemicals from ECM after decellularization, particularly detergents such as SDS

that penetrate into thick or dense tissues. Cytotoxicity is possible even at reduced agent concentrations and will inhibit or completely negate the beneficial properties of a cell-free ECM scaffold.Also this procedure was carried on using an immersion method with continue agitation.

2.3 Decellularization characterization

The decellularized tissue was characterized after one and two cycles of both the DET and SDS protocols in order to determine the optimum number of cycles required for the specific tissue. Firstly, histological analysis stained with hematoxylin and eosin (H&E) was performed. Hematoxylin stains the nucleic acids in blue and eosin stains proteins non-specifically in pink. The nuclei of a fresh tissue are stained in dark blue while cytoplasm and extracellular matrix are stained in different tone of pink. H&E represents a qualitative way of visualising the presence of nuclear material and is a valuable tool for understand the degree of decellularization by comparing fresh and decellularized tissue. Another staining used to further characterize the presence of DNA is DAPI (4',6diamidino-2-phenylindole), it is a florescent stain that emits blue light when bound to two double-stranded A-T rich region of the DNA. Others staining had been used to highlight different components of the ECM before and after decellularization such as Picro Serius Red staining for collagen , purple EVG staining for elastin, and Alcian Blue staining for GAG. 1cm^2 samples were fixed in formaldeide, embedded in paraffin, sections and then stained.

The DNA remaining in the tissues after decellularisation was assessed using a PureLink® Genomic DNA Mini Kit from Invitrogen. Three different samples were taken both for (sub)mucosa and whole intestine. The concentration of DNA present in the samples was then quantified with a nano-drop (NanoDrop 1000 Spectrophotometer by Thermo scientific®), which measures the absorbance of the light considering that DNA absorbance present a pick on 260 nm. Three measurements were taken for statistic liability. The measurement gives the averaged quantity of DNA present in the three tissue pieces expressed in ng of DNA per mg of wet tissue.

For the fabrication of the hydrogel it is important to conserve the extracellular matrix as much as possible to preserve the bioactivity of the native environment. The conservation of some of the ECM components such as collagen and GAGs was quantified. QuickZyme® Collagen assay kit was used to determine the quantity of collagen present in the (sub)mucosa and whole intestine in fresh tissue and after each cycle of decellularization for both DET and SDS protocols. Three samples of approximately 15 mg for each passage were taken for biological relevance, and each samples was run in duplicate. Small amounts of tissue were weighed, hydrolysed at 95 ° C in 6M HCl for 20 hours, for the breakage of all the peptide bonds and free

the amino acid in the solution. The samples were then placed into a 96-well plate and the assay buffer and the detection reagents were added, in that order. A small oven was used to incubate the plate at 60°C for an hour to make the hydroxyproline staining reaction occur. The plate was then cooled down to room temperature using ice and put into an infinite f200 plate reader from TECAN®, for the measure of the absorbance at 570 nm.

The relative content of collagen in the tissue, expressed in μg per mg of wet tissue, was derived from the quantification of the amount of hydroxiproline present in the samples. This is a modified amino acid that is almost only present in the triple helix of collagen. This methodology of quantification does not distinguish between different types of collagens and its degradation products, so it is not possible to quantify the different types of collagen which could be present in the samples

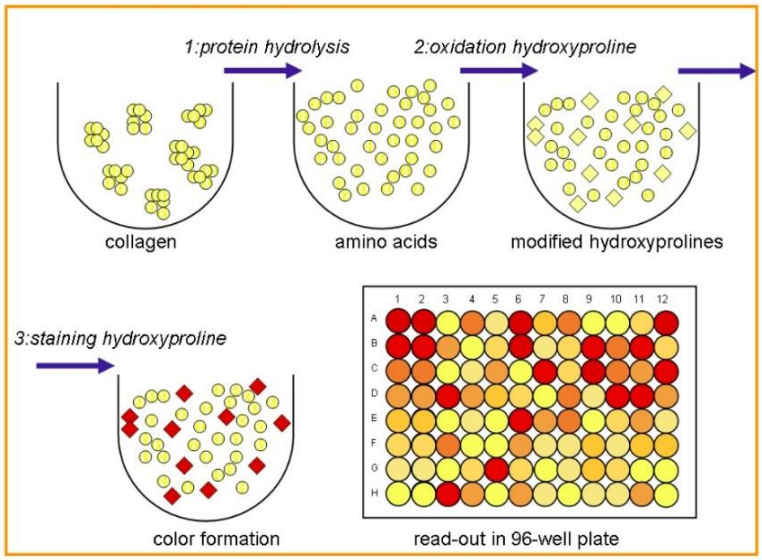

Figure 2.1. *Assay principle (from QuickZyme Total Collagen Assay manual updated version November 2012)*

Sulfated proteoglycans and glycosaminoglycans, (sGAG) quantification was analysed using the Blyscan ™ Sulfated Glycosaminoglycan Assay kit by Biocolor. The Blyscan Assay is a quantitative dye-binding that contains 1,9-dimethyl-methylene blue in an inorganic buffer, which also contains surfactants providing a specific label for the sulfated polysaccharide component of proteoglycans or the protein free sulfated glycosaminoglycan chains.
Prior the measurement of sGAG from tissue samples the sGAG were extracted using a papain extraction reagent, prepared with 50ml of a 0.2M sodium phosphate buffer, (Na_2HPO_4 –

NaH_2PO_4), pH 6.4 adding 400 mg sodium acetate, 200 mg EDTA, disodium salt ,40 mg cysteine HCl and to this introducing 250 ul of a papain suspension, containing about 5 mg of the enzyme. A Dissociation Reagent contains the sodium salt of an anionic surfactant was used to dissociate the sGAG-dye complex and enhance the spectrophotometric absorption profile of the free dye. Absorbance was measured at 650 nm and results were expressed in ug/mg of wet tissue.

In order to determine the effects of the decellularization methods on the surface structure of the Piglet whole intestine and (sub)mucosa intestine, three samples for each cycle of washing of DET protocol and for each day of SDS protocol underwent SEM analysis. Samples of dimension of 1cmm^2 were fixed in 2.5% glutaraldehyde. Samples were then fixed in 1% OsO4 in PBS for 15 min each, dehydrated in graded series of alcohol (30‑100%) baths for 15 min each. Samples were then critically point dried with hexamethyldisiloxane, mounted on studs, sputter coated, and stored in ad esiccator until imaged. SEM images were captured using a JEOL 6335F Field Emission SEM instrument with a backscatter detector.

2.4 ECM derived hydrogel methodology

After the decellularization the tissue obtain has to be processed to derive the desired gel. First step consist in the lyophilisation of the tissue by using a freeze-dryer which works by freezing the starting material and then reducing the pressure to allow the sublimation of the frozen water present in the sample from the solid phase to the gas phase. After two days of freeze-drying the samples are milled into a thin powder using a mini-mill (Thomas Wiley® model) with intermediate sized filter (mesh 40).
The powder has then been digested with an HCl-pepsin (Pepsin from porcine gastric mucosa from SIGMA® life science) solution. The solution is obtained by adding 1 mg of pepsin powder to each ml of HCl 0.1 M. 10 mg of ECM powder are digested in 1 ml of solution in order to obtain the digestion of the macromolecules that are present and transform them into a more soluble mixture of proteins. The volumes which were usually adopted are 10 ml or 5ml.
The samples were then positioned in a shaker at room temperature for 72 hours. After the digestion the samples were neutralized using NaOH (sodium hydroxide). A 10% volume of 10xPBS solution was used as buffer to facilitate the neutralization and to avoid overshooting. Drops of 5 M and 1 M NaOH were used for the neutralization and the pH changing were recorded with a pH-meter. All process was carried on ice to slow to gelation kinetic so to have a less viscous solution, in fact the diffusion of NaOH was easily reached in a less viscous solution since to avoid the formation of to many bubbles it was not possible to use a vortex. When reached pH of 7.4 gelation was then achieved by placing the neutralized solution in an incubator at 37° C (physiological temperature). Gel was derived from both (sub)mucosa and

whole intestine tissue for DET protocol and from whole intestine for SDS protocol to determine which of them would result in the most promising gel.

The initial concentration of 10 mg of ECM per ml was then diluted after the digestion to 8 and 6 mg per ml to verify the effect on the initial viscosity of the solution and on the rheological property of the gel. For all the dilution 1 x PBS was added to obtained the desired concentration.

2.5 Gel Characterization

Turbidity analysis and rheological tests were performed to determine the gelation kinetic and the viscoelastic properties of the obtained gel.
For turbidity analysis 200 µl of the neutralised gel were putted into a 96-well plate and placed in a plate reader pre-heated to 37 ˚C for a duration of 1 hour. The absorbance of the samples was measured at 450 nm once every minute for one hour. This technique is commonly used to determine the gelation kinetic of ECM derived hydrogel since the spectrometer has the ability to detect the change in turbidity between the viscous solution to the gel during the self-assembly of the molecules.
The results were then scaled from 0 (minimum absorbance) to 1 (maximum absorbance) to provide a normalized absorbance (NA) as shown in Eq. (2.1) where A is the absorbance at a given time, A_0 is the minimal absorbance and Amax is the maximum absorbance when the plateau is reached and the gelation is completed.

$$NA = \frac{A-Amin}{Amax-Amin} \tag{2.1}$$

The typical evolution of the curve when plotted as function of time consist in a sigmoidal curve with a lag time in which the absorbance is maintained nearly to 0 , followed by a phase of almost linear increase in absorbance and a plateau region when gelation is completed where the absorbance reaches its maximum value and is stationary.
The main parameters which were obtained from the normalized curve were the time of half gelation ($t_{1/2}$), which was the time required to reach the value of 50% of the normalized absorbance, the lag time (t_{lag}) was defined as the intercept of the linear region of the gelation curve with 0% absorbance, and the speed of gelation or the gelation rate (S) was defined as the maximum slope of the growth portion of the curve.

Viscosity, which represents the ability of a fluid to resist flow/deformation was measured using a Bohlin CVO120 stress-controlled rheometer with a 40 mm parallel plate geometry. It consist in a two parallel plates where the upper plate move applying a force F on the surface while the lower one stays stationary. The ration between the force and the plate area is called shear stress

σ measured in Pa. The shear rate represent the derivative of the ratio of the velocity v of the upper plate to the distance h separating the two plates, and correspond to the rate at which the fluid is deformed by the applied shearing force. It is expressed in reciprocal seconds [S^{-1}].

Dynamic viscosity η , is defined as the ratio between shear stress and the shear rate. One ml of each sample was placed between the two plates of the rhemoter before neutralization and at room temperature, and viscosity and shear stress were measured for a shear range of 10 to 150 [S^{-1}].

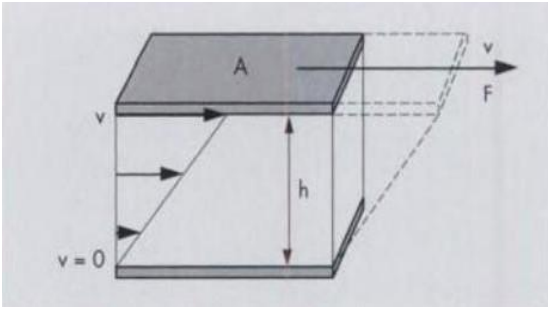

Figure 2.2. *Illustration of the two plate model.*

Oscillatory properties of ECM were determined using the same instrument. Pre-gel solution were first neutralized on ice and then placed between parallel plates separated by 1 mm gap. For each samples were used 3 ml of solution, and the plates of the rheometer were pre-warmed to 37°C.

A 60 min time course experiment was performed in which the samples were subjected to an oscillatory strain of constant maximum amplitude of 0.5 Pa at a constant angular frequency of 1 Hz with readings taken every 30 s. For stress-controlled rheometers the shear stress is applied as $\tau(t) = \tau_0(\sin(\omega t))$ and the resulting shear strain is measured as $\gamma(t) = \gamma_0(\sin(\omega t + \delta))$ for duration of 1 hour.

For a purely elastic material, the strain and stress waves are in phase ($\delta = 0°$) while a purely viscous response has the two waves out of phase by 90°($\delta = 90°$). Viscoelastic materials give rise to a phase-angle somewhere in between, which represent the lag between the applied stress wave and the resulting recorded sinusoidal strain response, with a phase shift is comprised in between $\delta = 0^{\circ}$ and $\delta = 90^{\circ}$ meaning that the material exhibits an intermediate behaviour between pure elastic and pure viscous.

Considering the type of material it is possible that a lag between the applied sinusoidal stress wave and the may occur, which is quantify as δ, the phase angle shift between the stress and the strain. For the called purely elastic solids, the strain is in phase with the stress ($\delta = 0^{\circ}$); the

maximum strain occurs when the applied stress is maximal. On the other hand, when the stress and the strain are out-of-phase ($\delta = 90^{\circ}$), it means that the material is purely viscous.[44]

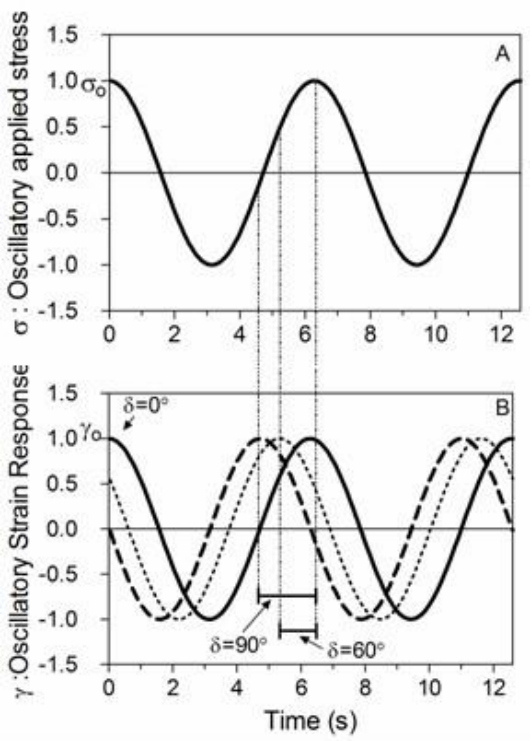

Figure 2.3. *Oscillatory applied stress, σ, and the resulting strain, γ, with three different phase angles δ.*

In small amplitude oscillatory shear measurements, the shear storage modulus, G′, loss modulus, G″, and loss factor, tan δ, are critical hydrogel properties which were monitored against time. Using the phase shift angle it is possible to derive two important quantities, the elastic (or storage) modulus, G', and the viscous (or loss) modulus, G". The relationship which links them to the phase angle is the tan(δ) = G"/G'. The loss factor, tan δ, is defined as G″/G′ and represent the link between the two moduli.

G′ measures the deformation energy stored during shear process of a test material (i.e. the stiffness of the material) and G″ is representative of the energy dissipated during shear by viscous friction through the material (i.e. the flow or liquid-like response of the material). If G″ > G′ (tan δ > 1), the sample behaves more like a viscous liquid while, conversely, when G′ > G″, and, thus, tan δ < 1, the sample behaves more like an elastic solid. Before gelation, as the solution is expected to have a more liquid-like behaviour, the viscous modulus should be larger than the elastic modulus. As the temperature rises inside the solution, the elastic modulus is supposed to increase and become larger than the viscous one, as the solution should start

behaving more like a solid than a liquid. The intersection of the two modulus curves is often considered to be the point where the solution turns into a gel [45].

2.6 Hydrogel cytotoxicity

Cytotoxicity of the hydrogels was studied by an indirect techniques MTT colorimetric assay for day 1 and day 5. (3[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich). The MTT assay measures the ability of metabolic active cells to form blue formazan crystals through cleavage of the tetrazolium ring of MTT. For this, 3T3-J2 mouse embryonic fibroblast cells were used, which were cultured using Penicillin-Streptomycin+Hyclone Animal Sera, HyClone SH30072.03, Bovine medium and Calf Serum, Iron Supplemented+ DMEM, high glucose (seeded onto well plates). ECM hydrogel used for testing was derived from (sub)mucosa only, because it was considered to represent the best candidate, and sterilized in the gamma irradiator 16 hours over night.

Different conditions were tested: Seeding cells on top of gel using a concentration of 8 mg/ml. Incorporating cells into the gel using a concentration of 8mg/ml, Coating the plate with ECM suspension concentration 1mg/ml, Mixing ECM suspension in the culture medium with concentration of 1mg/ml, Seeding Cells alone, Placing gel with no cells in each well. To account for cell viability the absorbance was measured at 555 nm. For all the seeding the cell density used was 6666 cells for each 96 plate well. The volume of gel per well was 50µl.

2.7 Organoids Test

Organoids culture has been preliminary tested at Crick Institute with Laween Meran and Sang Eun Lee. The gel was irradiated overnight and then neutralized to fisiological pH. Was then placed into well plates into droplets of 40 µl and the organoids, which were cultered by Laween Meran, were addeded and then placed into the incubator for half an hour in order to obtain the polymerization of the gel. The gel that has been used is 8ml/ml from mucosa intestine. Images from day 0 3 and 6 were taken in order to observe the behavior and morphology of the organoids in the new gel

Chapter 3

Results and Discussion

In this chapter are presented all the results of the characterizations. Starting with the decellularization, and proceeding with the turbidometric, viscosity and rheological results. Lastly are presented the tests on citocompatibility and organoids culture.

3.1 Piglet intestine decellularization

A protocol for effective decellularization of whole intestine and (sub)mucosa intestine was identified with the use of a detergent enzymatic treatment. For the whole intestine also an ionic detergent treatment alone was successfully obtained.

3.1.1 Efficacy of the decellularization

The term decellularization has not been defined by quantitative metrics, but some general minimal criteria of investigation are consider sufficient to investigate the degree of decellularization to avoid adverse cells and host response. These are based upon the findings of studies in which an *in vivo* constructive remodelling response has been observed and adverse cell and host responses have been avoided, and which are the lack of visible nuclear material in tissue sections stained with DAPI or H&E and an amount <50 ng of DNA per g ECM wet weight. [22]
The attention on nucleic material is justified because DNA is directly correlated to adverse host reactions, is present all across tissue and cell types, is easily assayed, and provides a general index for other cell residues within ECM. [46]

A macroscopically observation of the piglet intestine change in tissue colour suggested a good level of decellularization in the samples. In fact it was possible to observe a completely change in colour in the decellularized tissue compared to the native one. Figure 3.1 shows how fresh tissue appear as dark pink while decellularized tissue appear transparent.
The degree of decellularization was then qualitative verify using haematoxylin and eosin staining to observe the presence or absence of nuclei content in both whole intestine and (sub)mucosa intestine. Here are presented the staining for fresh tissue and tissue after one cycle and two cycle of DET protocol.

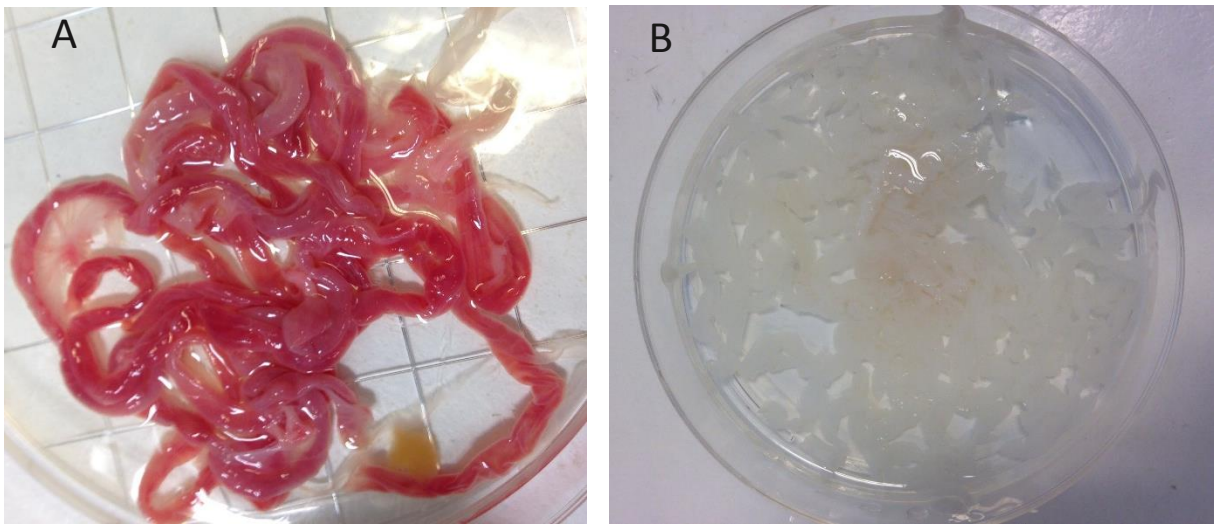

Figure 3.1. *A) Fresh tissue piglet whole intestine B) Decellularized tissue piglet whole intestine*

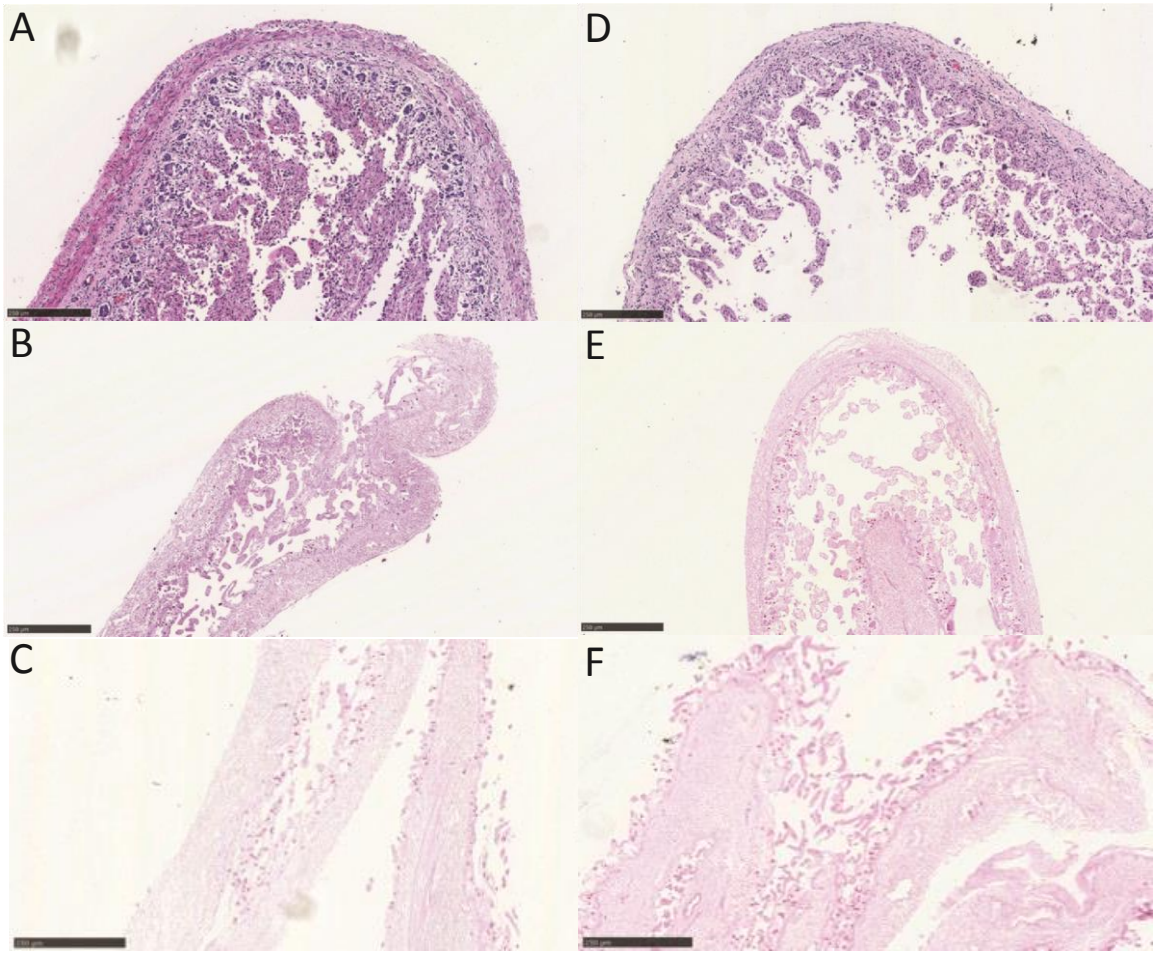

Figure 3.2. *Hematoxylin and eosin staining of the A) whole intestine fresh tissue, B) decellularized whole intestine after one cycle, and C) decellularized whole intestine after two cycles, D) mucosa intestine fresh tissue, E) decellularized mucosa intestine after one cycle, and F) decellularized mucosa intestine after two cycles. It shows the efficacy of decellularization. (Black bars = 250 μm).*

A clear difference between fresh tissue and the washed one was observed as result from H&E (image 3.2.), but since was not easy to observe the complete nuclei removal, corresponding to the dark blue circles, DAPI staining was also performed. From DAPI staining (fig 3.3) it was possible to confirm the absence of nuclei content since no light blue nuclei were visible from the images.

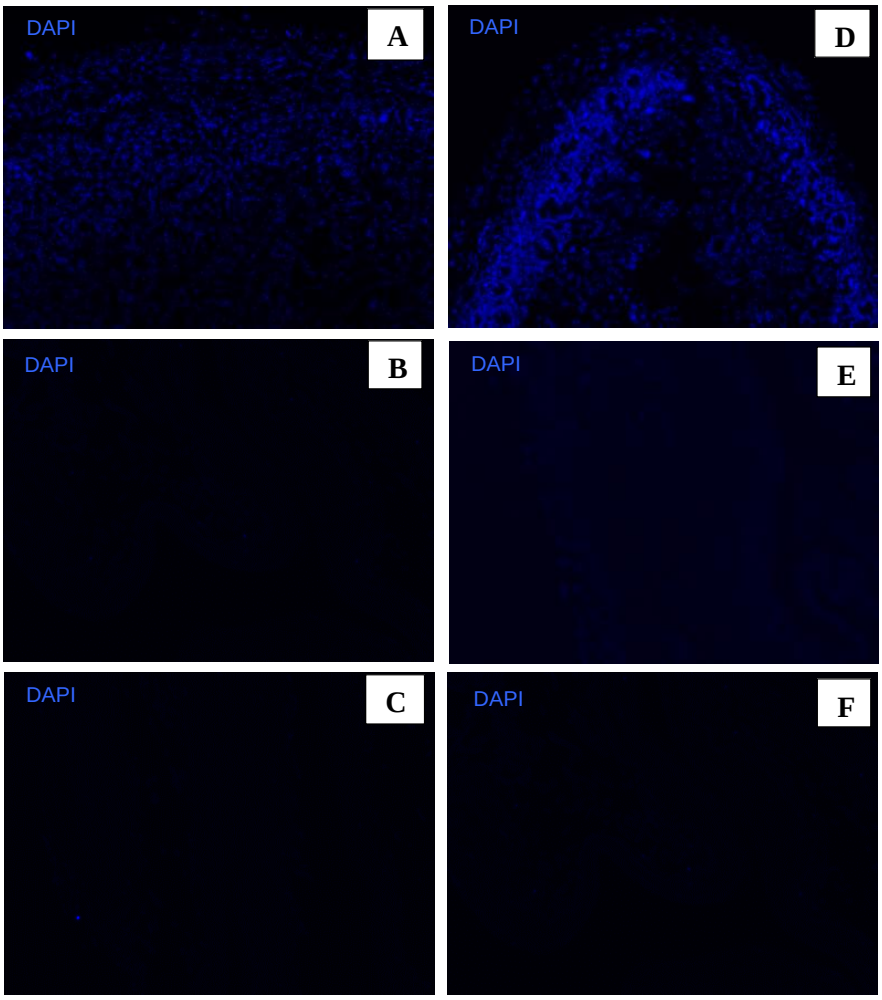

Figure 3.3. *DAPI stainings of the whole intestine A) before and B) after one cycle of decellularisatin,C) after two cycle of decellularization. D)DAPI staining for fresh mucosa intestine and E)after one cycle of DET F) after two cycle of DET.*

To quantify this results DNA quantification assay was performed, and from the results (image 3.4) it was possible to observe that there was a significant diminishing in the amount of DNA in the decellularized tissue compared to the fresh one. However there was no significant difference in the DNA content in the tissue after the first and second cycle. Taking in

consideration this result, the overall low levels of DNA present in decellularized tissue, and the results of the H&E and DAPI staining, the optimal number of decellularization cycles for piglet both whole intestine and (sub)mucosa was established as one.

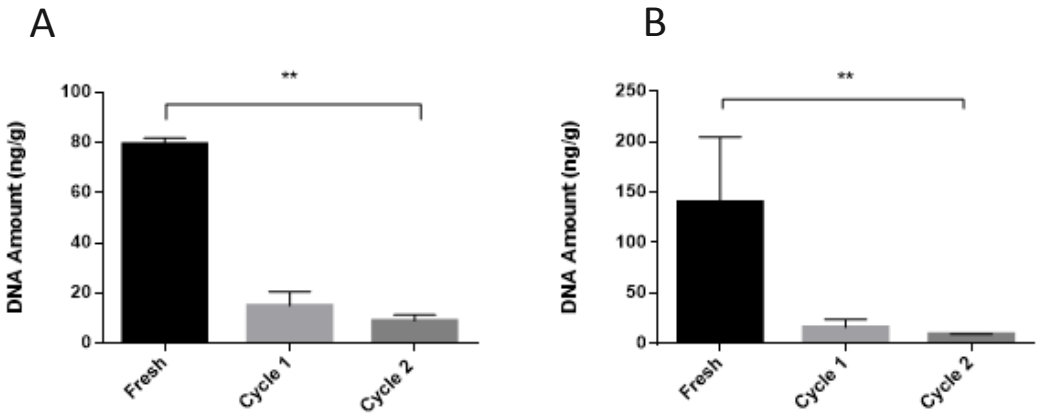

Figure 3.4. *A) DNA content of piglet whole intestine for fresh tissue and after one cycle and two cycle of DET protocl. B) DNA content of piglet mucosa intestine for fresh tissue and after one cycle and two cycle of DET protocl. Results are expressed as mean values and standard deviation [ng/g of wet tissue].It is possible to observe a significant($p<0.01$) decrease in the DNA amount after one cycle of DET, while nosignificant difference is observed between cycle one and cycle 2 ($p>0.05$).*

The same procedure was carried out for SDS protocol for whole intestine, H&E and DAPI showed that also in this case there was no nuclei content left after one day and two day of decellularization. From DNA quantification assay it was possible to quantify the remnant content of DNA for the different samples. Results (fig. 3.5) showed that DNA concentration was markedly less than those presents in the native tissue, but no significant difference between one day and two day of SDS washing was found, meaning that one day of this protocol was sufficient to obtain an efficient decellularization.

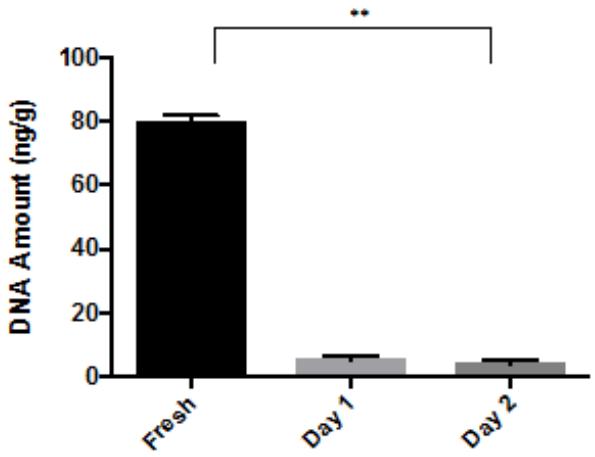

Figure 3.5. *DNA content of piglet whole intestine for fresh tissue and after one day and two day of SDS protocol. Results are expressed as mean values and standard deviation [ng/g of wet tissue]. It is possible to observe a significant ($p<0.05$) decrease in the DNA amount after one day of SDS, while no significant difference is observed between day one and day 2 ($p>0.05$).*

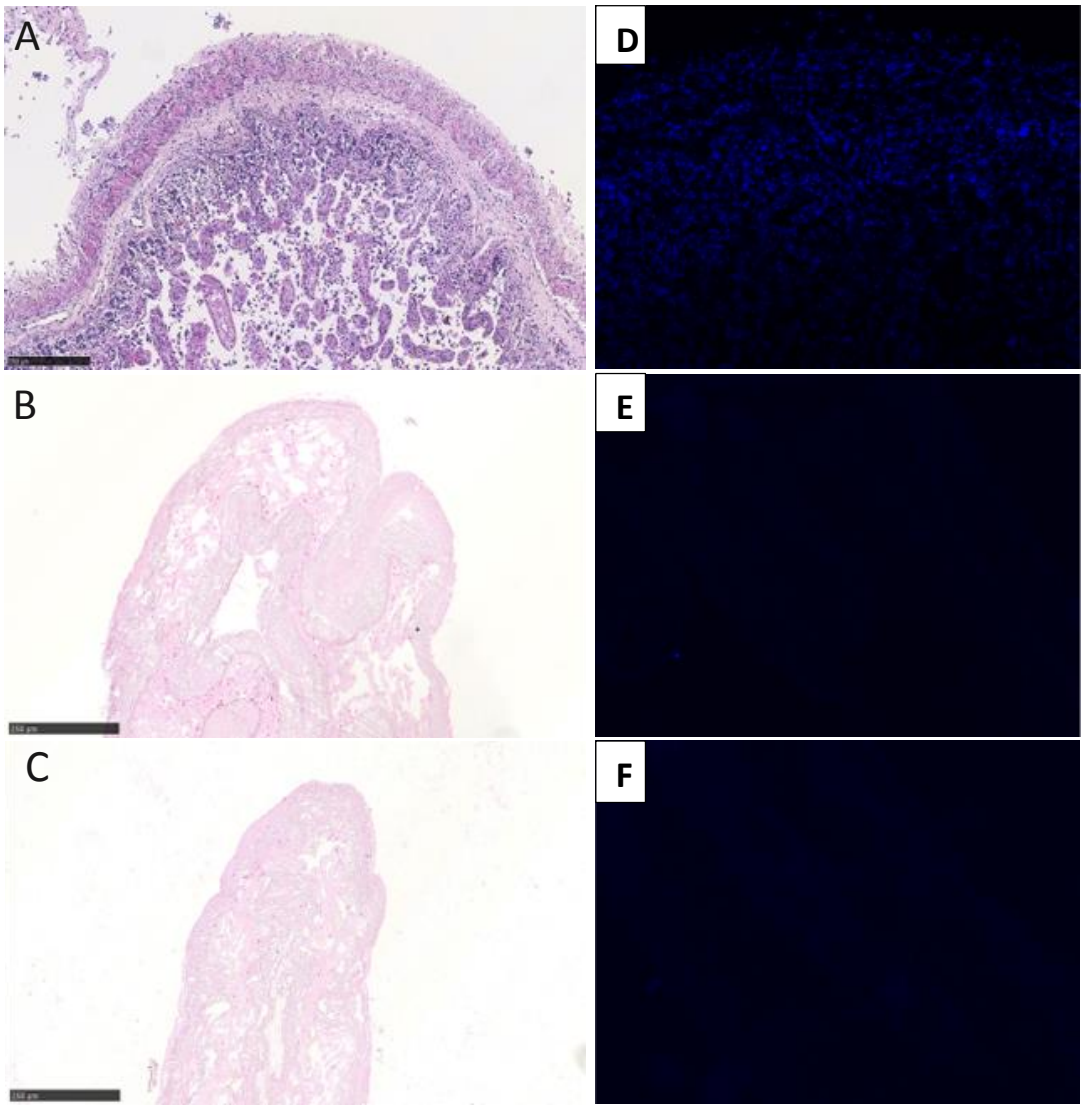

Figure 3.6. *Hematoxylin and eosin staining of the A) whole intestine fresh tissue, B) decellularized whole intestine after one day, and C) decellularized whole intestine after two day of SDS protocol. DAPI staining of the whole intestine D) before and E) after one day of SDS, F) after two day of SDS. Both stainings show the efficacy of decellularization. (black bars = 250 μm).*

3.1.2 Conservation of the ECM

In order to further characterize the structure of the decellularized tissue and assess the effects of the detergent-enzymatic treatment to the ECM of the intestine (sub)mucosa and whole intestine some ECM components such as collagen, elastin end GAGs were evaluated.

Picro Serius Red which stains collagen in red showed that collagen was still present in the decellularized tissue. Using Verhoeff's Van Gieson (EVG) staining which stains elastin in purple it was possible to confirm the presence of elastin in the decellularized tissue, while Alcian Blue staining confirmed the presence of GAGs after 1 cycle of DET

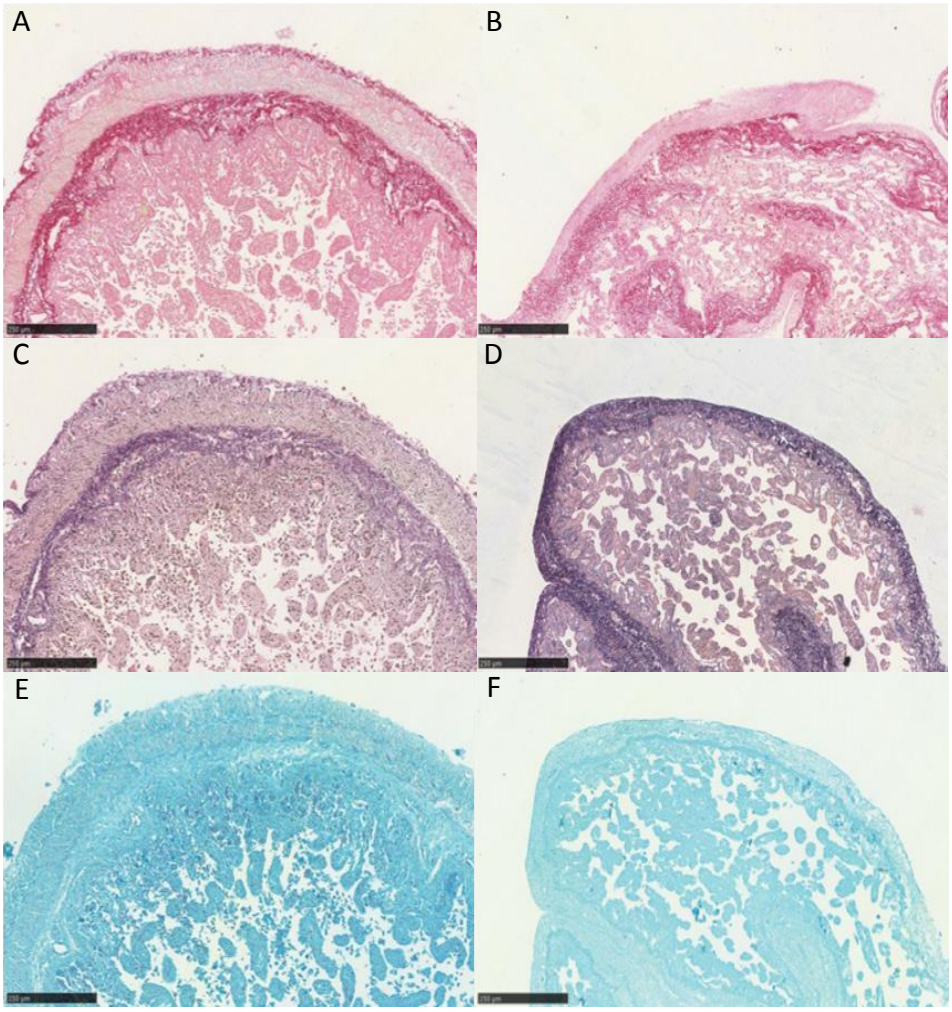

Figure 3.7. *Stainings of whole intestine extracellular components before and after the first cycle of decellularisation with DET protocol.(A) and (B) Picro Serius Red shows collagen in red before and after decellularization, (C) and(D) EVG staining shows elastin in purple before and after decellularization, (E) and (F) Alcian Blue shows GAG in cyan before and after decellularization. These stainings show that some the main components of the ECM are still present after one cycle of decellularization (black bars = 250μm).*

Collagen (Fig.3.7 A,B) and elastin (Fig. 3.7 C,D) staining appear to maintain their intensity and their preservation after 1 cycle of DET, whereas GAGs were gradually depleted by the decellularization treatment (Fig. 3.7 E,F).

In line with the histological findings also a quantitative assay was performed for Collagen and GAGs content. The quantitative assay showed that extracellular matrix collagen was preserved after the decellularization and its content (collagen/wet tissue weight) was significantly enhanced with an increasing number of DET cycles ($P < 0.05$; Fig. 3.8 A). In contrast, the amount of GAG declined progressively in the decellularized intestinal tissue as demonstrated by staining ($P < 0.05$; Fig. 3.8 B).

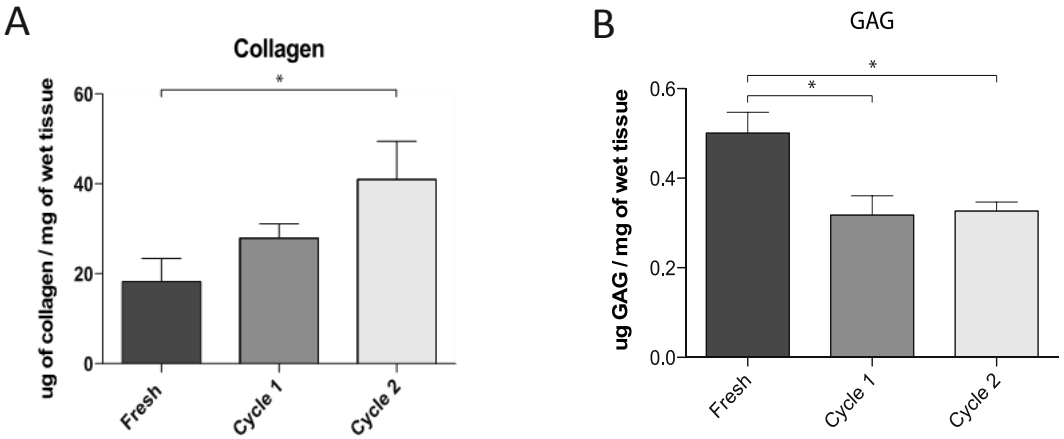

Figure 3.8. *A) Collagen content of piglet whole intestine for fresh tissue and after one cycle and two cycle of DET protocol. Results are expressed as mean values and standard deviation [ug/mg of wet tissue]. It is possible to observe a significant ($p<0.05$) increase in the collagen amount after one cycle of DET. B) GAGs content of piglet whole intestine for fresh tissue and after one cycle and two cycles of DET. A significant ($p>0.05$) decrease is observed compared to fresh tissue and cycle one and two.*

From the (sub)mucosa staining it was possible to observe the same preservation of Collagen and Elastin after one cycle of DET, while a decrease in the relative amount of GAGs is perceived. Quantitative assay showed also in this case a significant ($p<0.05$) increase of the amount of collagen in the tissue after the first and the second cycle of DET while GAGs content appeared lowered by the treatment.

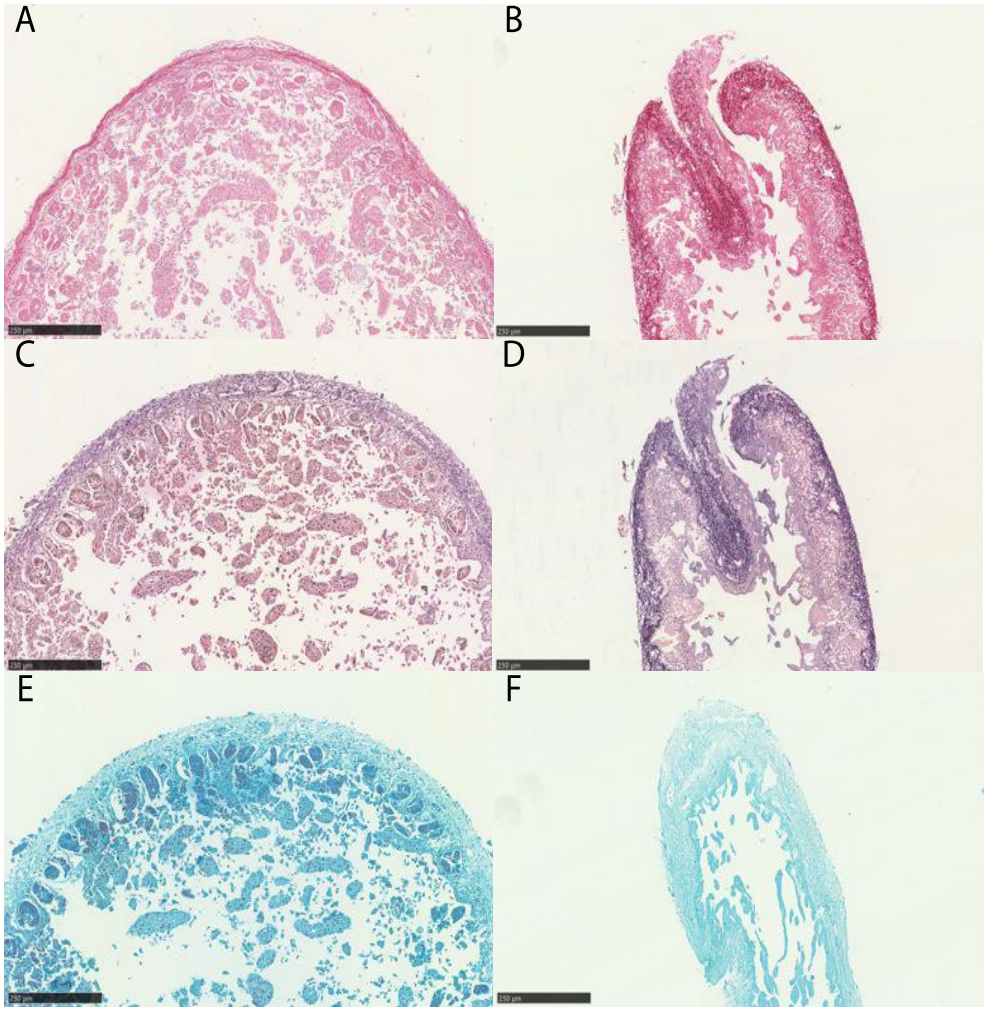

Figure 3.9. *Stainings of mucosa intestine extracellular components before and after one cycle of decellularization with DET protocol. A) and B) Picro Serius Red shows collagen in red before and after decellularization, C) and D) EVG staining shows elastin in purple before and after decellularization, E) and F) Alcian Blue shows GAG in cyan before and after decellularization. These stainings show that some the main components of the ECM are still present after the treatment (black bars = 250μm).*

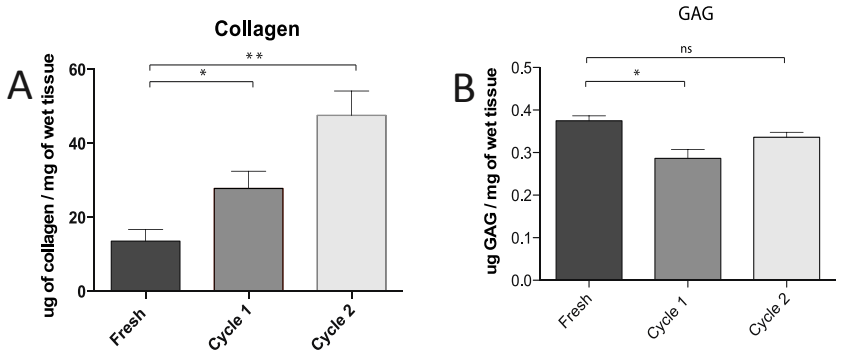

Figure 3.10. *A) Collagen content of piglet mucosa intestine for fresh tissue and after one cycle and two cycle of DET protocol. Results are expressed as mean values and standard deviation [ug/mg of wet tissue]. It is possible to observe a significant ($p<0.05$) increase in the collagen amount after one cycle of DET. B) GAGs content of piglet whole intestine for fresh tissue and after one cycle and two cycles of DET. A significant ($p>0.05$) decrease is observed compared to fresh tissue and cycle one and two.*

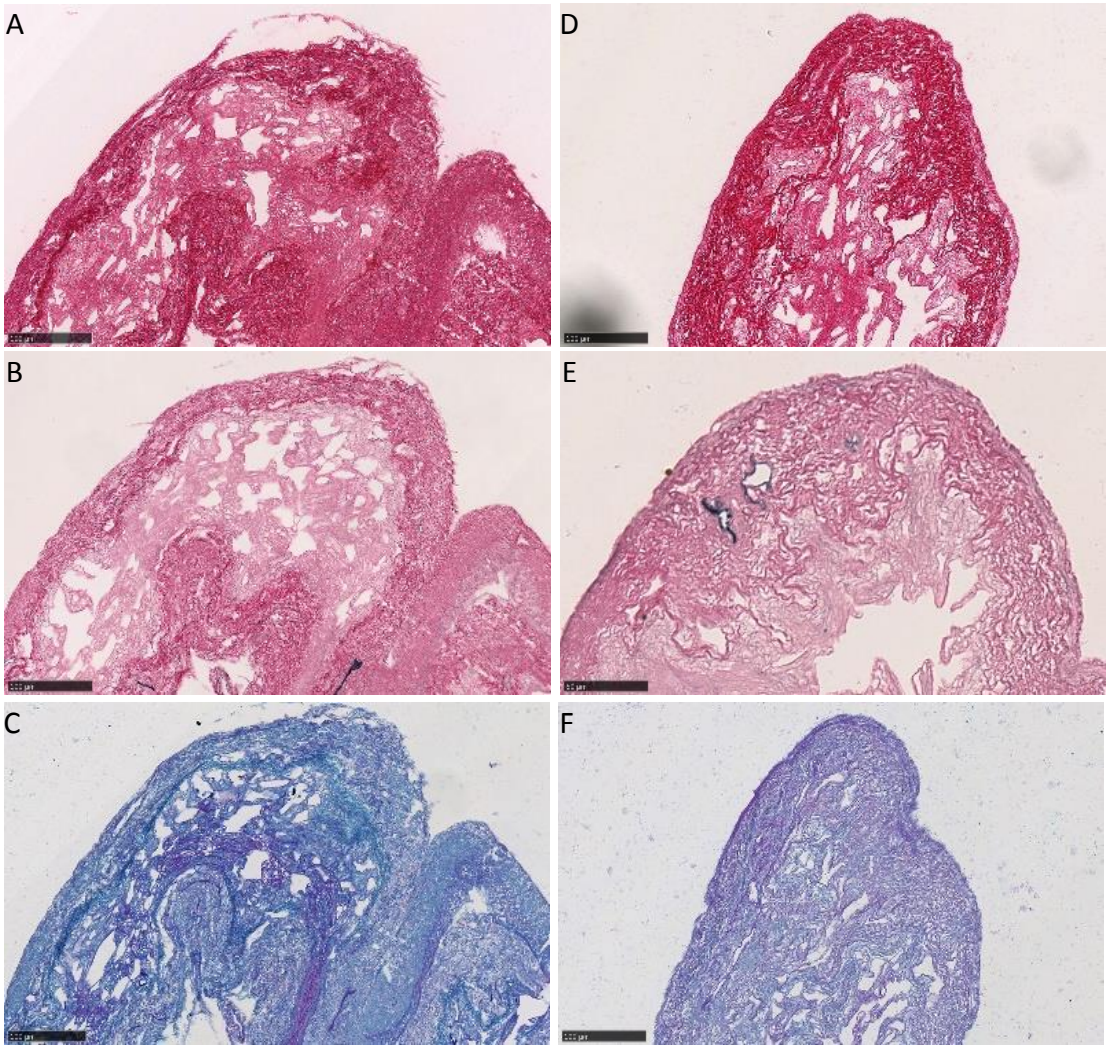

Figure 3.11. *Stainings of whole intestine extracellular components before and after one cycle of decellularization with SDS protocol. A) and B) Picro Serius Red shows collagen in red before and after decellularization, C) and D) EVG staining shows elastin in purple before and after decellularization, E) and F) Alcian Blue shows GAG in cyan before and after decellularization. These stainings show that some the main components of the ECM are still present after the treatment (black bars = 250μm).*

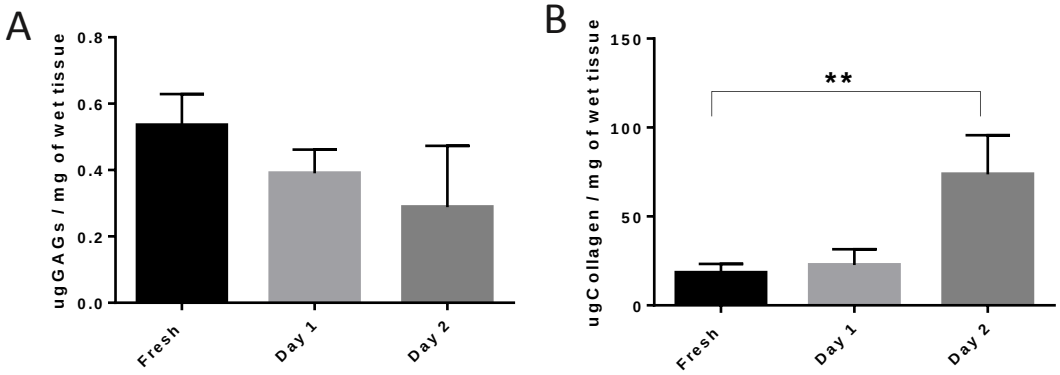

Figure 3.12. *A) GAGs content of piglet mucosa intestine for fresh tissue and after one day and two day of SDS protocol. Results are expressed as mean values and standard deviation [ug/mg of wet tissue]. A significant ($p>0.05$) decrease is observed compared to fresh tissue and day one and two.. B) Collagen content of piglet whole intestine for fresh tissue and after one day and two days of SDS. It is possible to observe a significant ($p<0.05$) increase in the collagen amount after one cycle of SDS*

For SDS treated samples collagen, elastin and GAGs staining exhibited their preservation after 1 day, (Fig. 4D). GAGs staining appear to be lowered by the treatment, while no particular difference was observed for the other stainings. Quantitative assay showed a significant increase (p value > 0.05) in the collagen content in the decellularized tissue (fig. 3.12) caused by the fact that it is one of the main component present in the ECM and its relative concentration increases. On the other hand no statistical difference is observed between the different samples in the content of GAGs (Pvalue > 0.05).

3.1.3 SEM results

SEM of the intestinal acellular matrix showed preservation of the micro and ultra-structural characteristics of the native tissue and confirmed the absence of cells. In particular, analysis of the luminal surface of the matrix after 1 cycle and cycle 2 of DET revealed the presence of leaf-shaped villi and crypts at their bases, meaning that they were not taken off during the washing cycle.

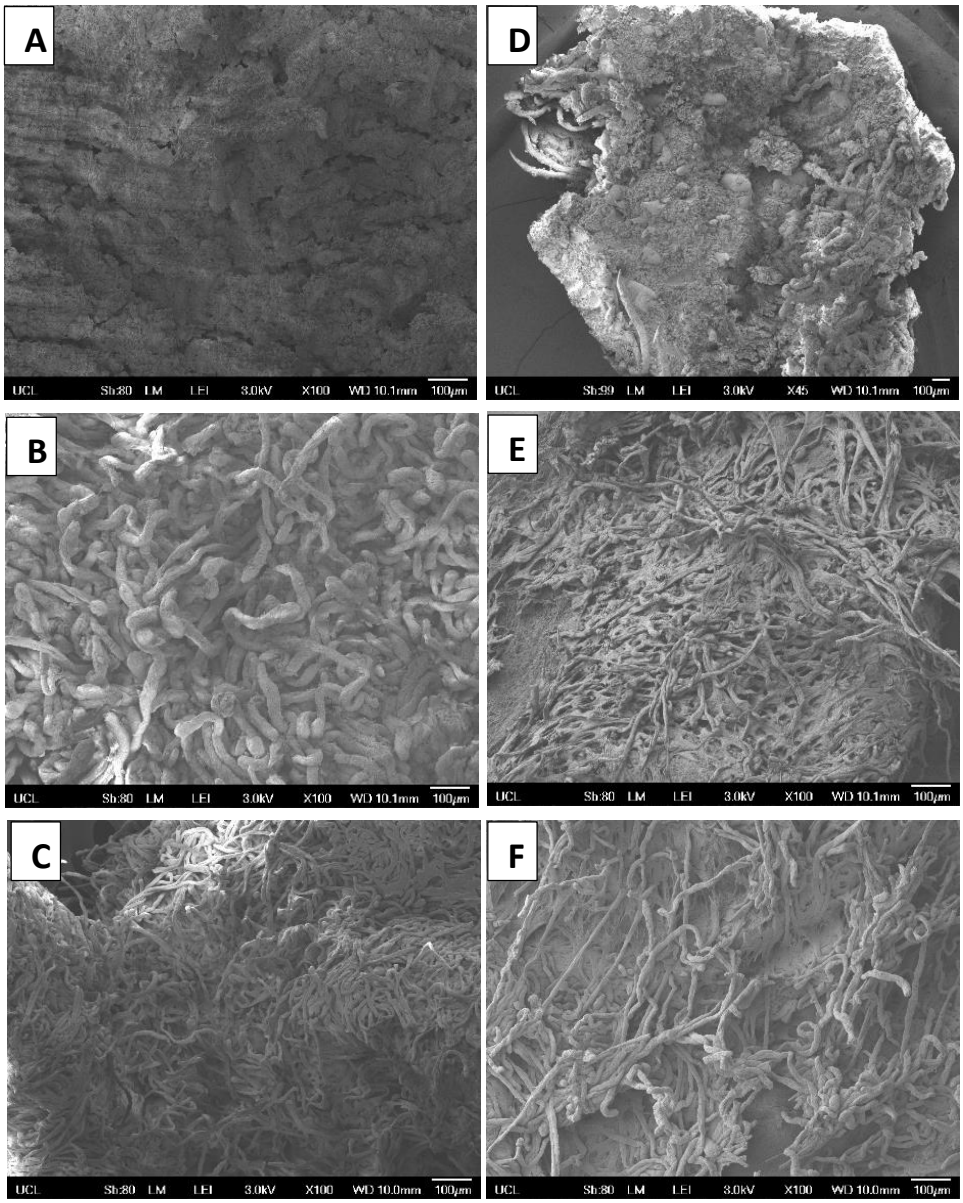

Figure 3.13. *SEM images from A) fresh whole intestine, and B) after 1 cycle and C) 2 cycle of DET. D)Mucosa intestine fresh tissue and E) after one cycle and F)two cycle of DET. Scale (black bars = 100µm).*

SEM images for SDS treatment revealed the presence of native architecture after 1 day and 2 day of decellularization, with the removal of cellular component. Any visible difference was observed between this sample and the same obtained from DET protocol.

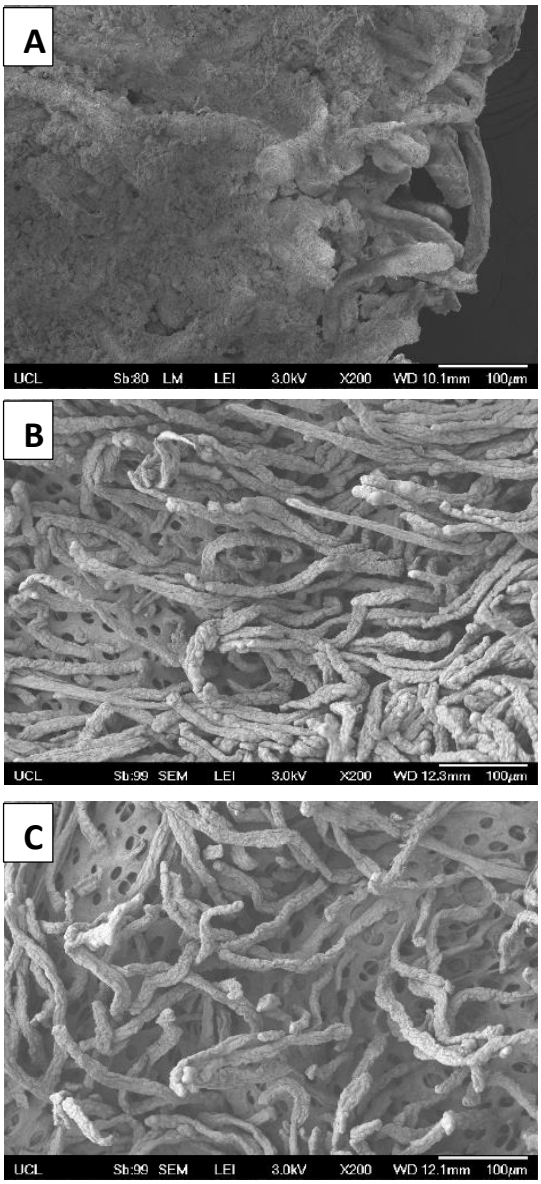

Figure 3.14. *SEM images from A) fresh whole intestine, and B) after 1 cycle and C) 2 cycle of SDS protocol. (black bars = 100µm).*

3.2 Gel Characterization

Different way of obtaining a thin powder were tested starting from the use of a mesh of 20 with mini mill machine, then a different size of the mesh which was 40 and 60, lastly immersion of the samples into liquid nitrogen followed by the use of mortal and pestle. The best method which was chosen was the use of an intermediate mesh of 40 and leaving the sample for a longer time in the mini mill machine until the powder obtained was observed to be as homogenous and thin as possible (fig 3.15 A).

The powder was then digested for three days and neutralized. The solution containing digested and neutralized ECM powder has been placed for half an hour in the incubator at 37°C in a 24 well- plate. After having realized it was possible to successfully obtain a gel with this procedure using ECM for both whole intestine and (sub)mucosa from DET protocol turbidometric and rheological properties of the ECM intestine hydrogel were evaluated for different concentration. SDS analysis have been carried on only for concentration of 10 mg/ml of powder in the digested solution.

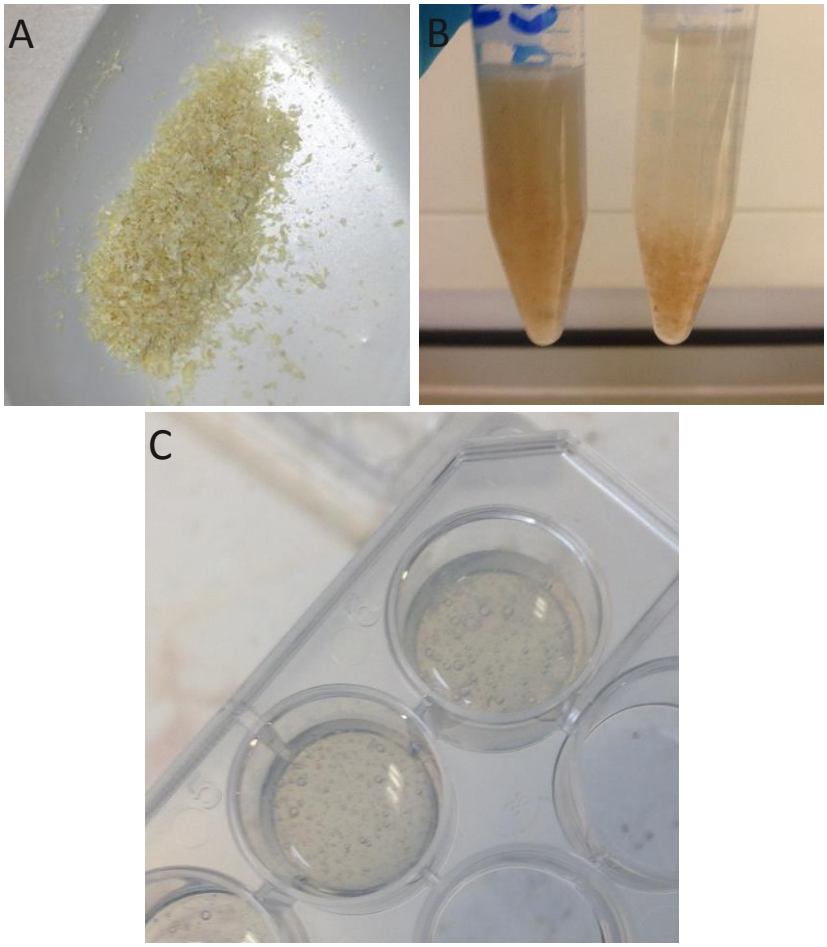

Figure 3.15. *A) thin powder of ECM obtained after one cycle of DET. B) solution containing HCl 0.1 M and pepsin for the digestion of the powder, and C) gel obtained after placing the neutralized solution into the incubator for half an hour.*

3.2.1 Thurbidometric analysis

The results from the turbidity measurements are presented in Fig. 3.16 and 3.17. For all the cases except whole intestine concentration of powder 6 mg/ml was possible to observe that absorbance was increasing with time until a plateau was reached. For concentration 6 mg/ml for whole intestine no sigmoidal curve was observed while the absorbance remained stationary. The value of the final absorbance was used to normalize the data and to define $t_{1/2}$ which is the estimated value required for gelation.

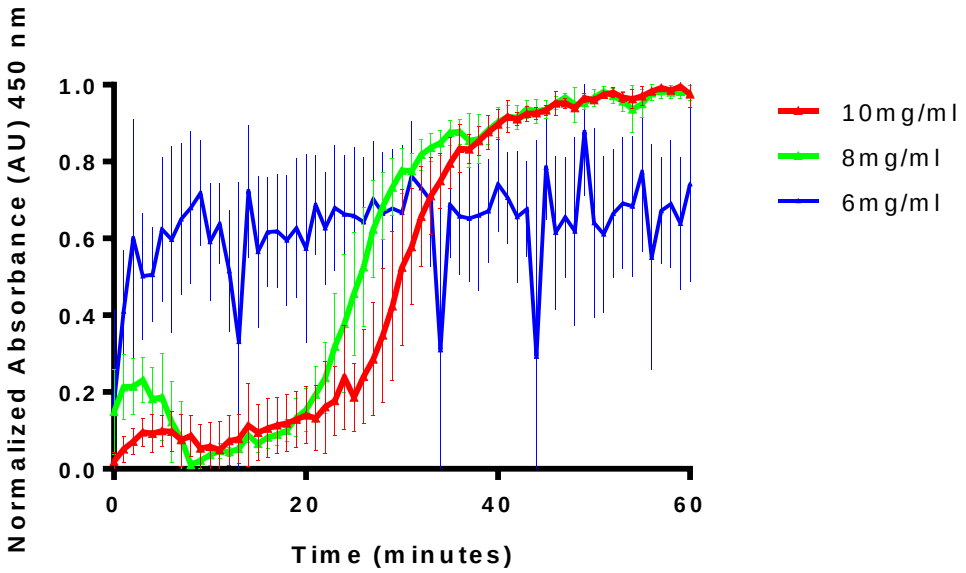

Figure 3.16. *Normalized absorbance at 450 nm for whole intestine derived hydrogel at different dilution 10 mg/ml, 8 mg/ml, 6 mg/ml. ECM was derived using DET protocol.*

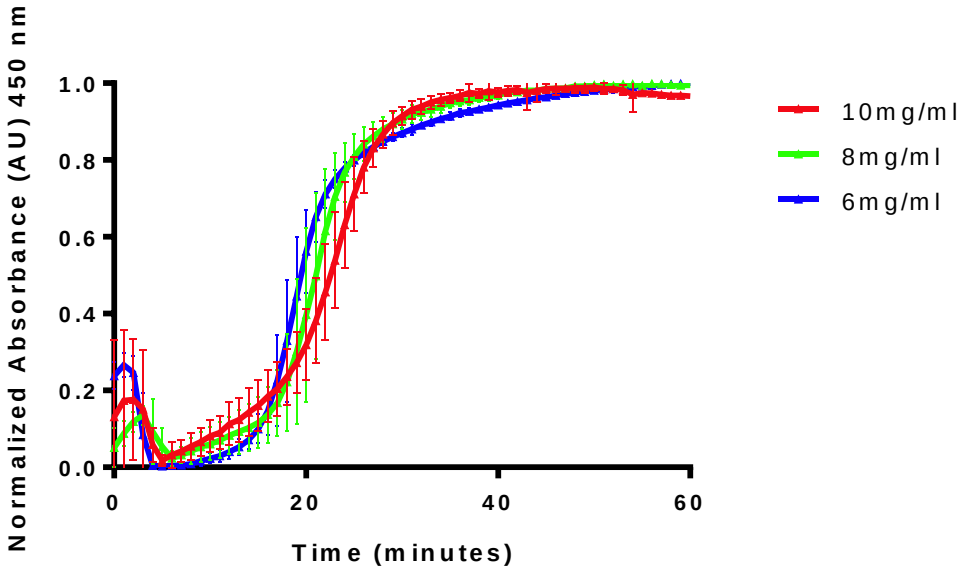

Figure 3.17. *Normalized absorbance at 450 nm for mucosa intestine derived hydrogel at different dilution 10 mg/ml, 8 mg/ml, 6 mg/ml. ECM was derived using DET protocol.*

Turbidometric analysis were carried on considering that during collagen I gelation, turbidity increase consequent to the fact that a nearly transparent solution of monomers develops into fibrils that scatter a significant amount of light.[47] The in vitro self-assembly of collagen monomers into fibrils has been well studied turbidimetrically, with the mechanisms of fibre network formation clearly elucidated[47]. This model reported a sigmoidal increase in turbidity during collagen self-assembly, with a lag phase of near zero turbidity followed by a growth phase with quickly increasing turbidity[48 47]. There is a starting phase of nucleation, which is the aggregation of soluble collagen particles into nuclei, where the lag period occurs, and then a phase where growth of the nuclei into fibrils predominate the nucleation.[47]

Turbidity measurements showed that both whole intestine and (sub)mucosa intestine derived hydrogel exhibited sigmoidal gelation kinetics consistent with a nucleation and growth mechanism, broadly consistent with collagen self-assembly model.

Although the mechanism and components responsible for gelation are unknown, the high content of soluble collagen in the derived extracellular matrix would suggest that gelation is largely due to the presence of self-assembling collagen molecules.

Of course the resultant gel is different to pure collagen most likely due to the interaction between the different components retained in the decellularized tissue, such as glycosaminoglycans, different types of collagen (III and IV) and other molecules which can modulate collagen self-assembly.[49]

The lag time (tlag), defined as the intercept of the linear region of the gelation curve with 0% absorbance, and the gelation rate (S) ,defined as the maximum slope of the growth portion of the curve were derived and results are presented in tab. 3.1 and 3.2.

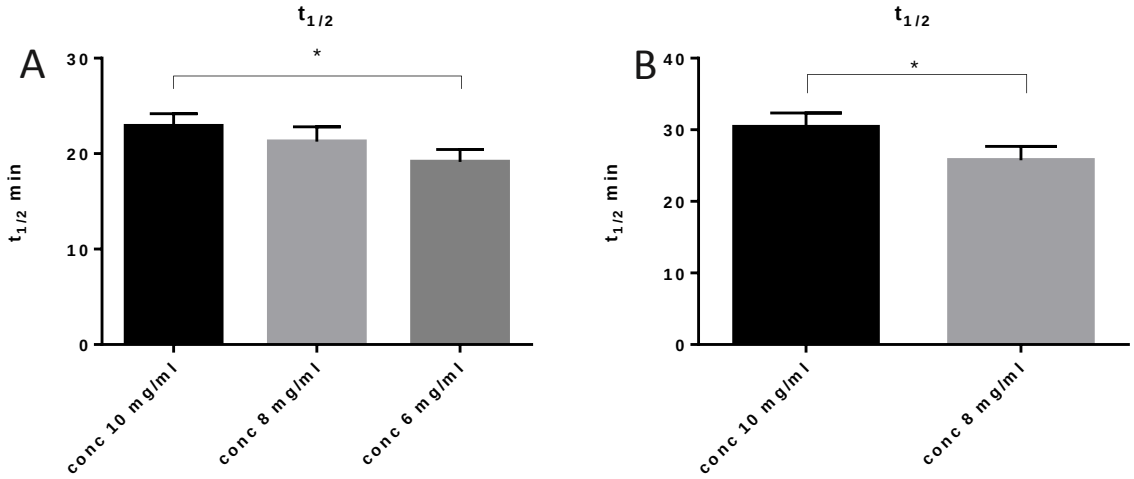

Figure 3.18. *Comparison of half gelation time for A) whole intestine at concentration of ECM powder of 10 mg/ml and 8 mg/ml, and B) comparison between half gelation time for mucosa intestine at concentration 10 mg/ml, 8 mg/ml and 6 mg/ml.*

Table 3.1. *Half gelation time, tlag and gelation speed are presented for the different dilution of whole ECM derived hydrogel*

Whole Intestine	**$t_{1/2}$**	**t_{lag}**	**S**
Conc. 10 mg/ml	30.38 ± 0.98	24.20 ± 1.12	0.093 ± 0.003
Conc. 8 mg/ml	25.75 ± 0.96	20.72 ± 0.70	0.11 ± 0.01

Table 3.2. *Half gelation time, tlag and gelation speed are presented for the different dilution of mucosa ECM derived hydrogel*

Mucosa Intestine	**$t_{1/2}$**	**t_{lag}**	**S**
Conc. 10 mg/ml	22.88 ± 0.65	16.63 ± 0.94	0.08 ± 0.01
Conc. 8 mg/ml	21.25 ± 0.77	16.70 ± 0.70	0.12 ± 0.01
Conc. 6 mg/ml	19.13 ± 0.65	16.10 ± 0.67	0.16 ± 0.01

From the results it was possible to observe that there was no significant difference in the $t_{1/2}$ between the different concentrations from dilution 10 to 8 in mucosa intestine. But there was a relevant difference between concentration dilution 10 and 6 in mucosa intestine.

Moreover comparing the Whole intestine and the (sub)mucosa it was possible to observe a relevant difference between the gelation times, in fact mucosa seemed to have a lower gelation time and a lower t_{lag}.

This highlighted that the gels derived from the (sub)mucosa did not gel faster compared to whole intestine one but that the gelation process started earlier.

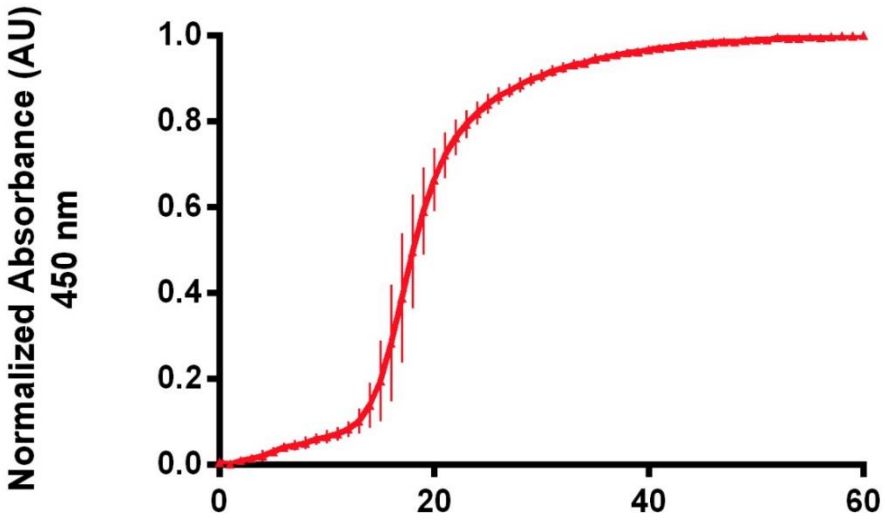

Figure 3.19. *Normalized absorbance at 450 nm for mucosa intestine derived hydrogel at dilution 10 mg/ml. ECM was derived using SDS protocol.*

From SDS protocol it was possible to observe the same sigmoidal curve of the DET protocol, as shown in fig. 3.19. This curve may be associated to the nucleation and formation of a fibrolous structure of collagen as well as previous reported. The $t_{1/2}$ obtained is observed to be markedly less than the one of the DET whole intestine conc. 10 mg/ml as well as the t_{lag} meaning that the process hypotetize takes markedly less time for this sample.

Table 3.3. *Half gelation time, tlag and gelation speed are presented for the different dilution of whole ECM derived hydrogel from SDS protocol*

Whole intestine	**$t_{1/2}$**	**t_{lag}**	**S**
Conc. 10 mg/ml	17.88± 0.5543	14.34± 0.58	0.123 ± 0.002

3.2.2 Viscosity Results

Viscosity was measured as a function of the shear rate before gelation occurred, with a shear rate increasing from 0 to 150 s^{-1}.

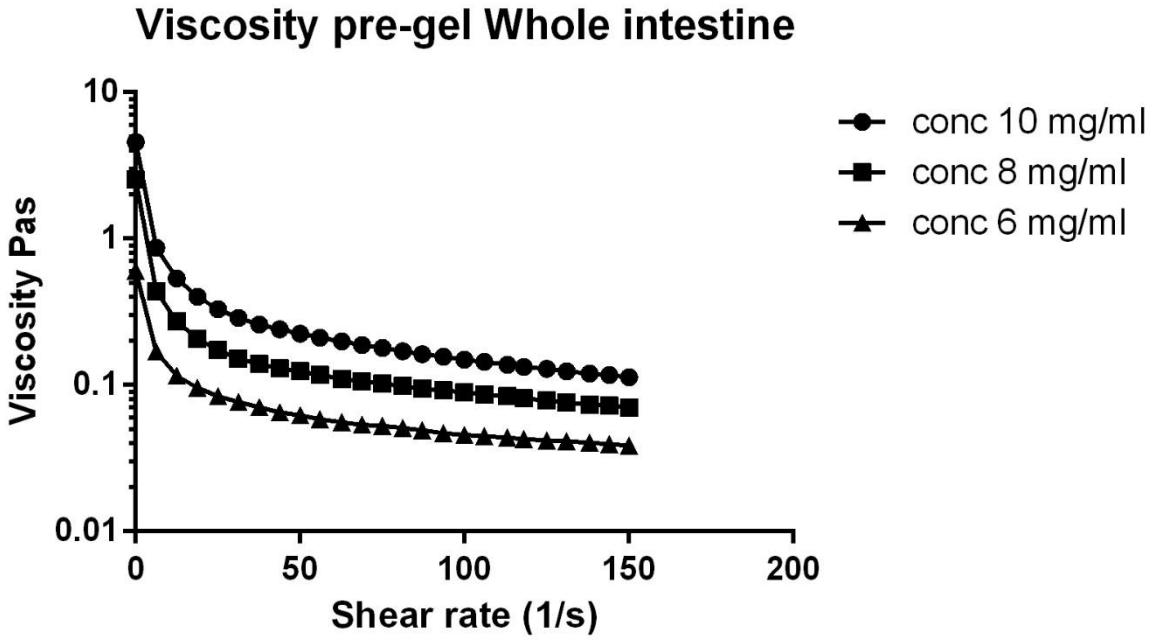

Figure 3.20. *Viscosity as function of shear rate for pre-gel whole intestine digested solution[Pas] for concentration of 10mg/ml , 8 mg/ml and 6 mg/ml obtained from DET decellularization.*

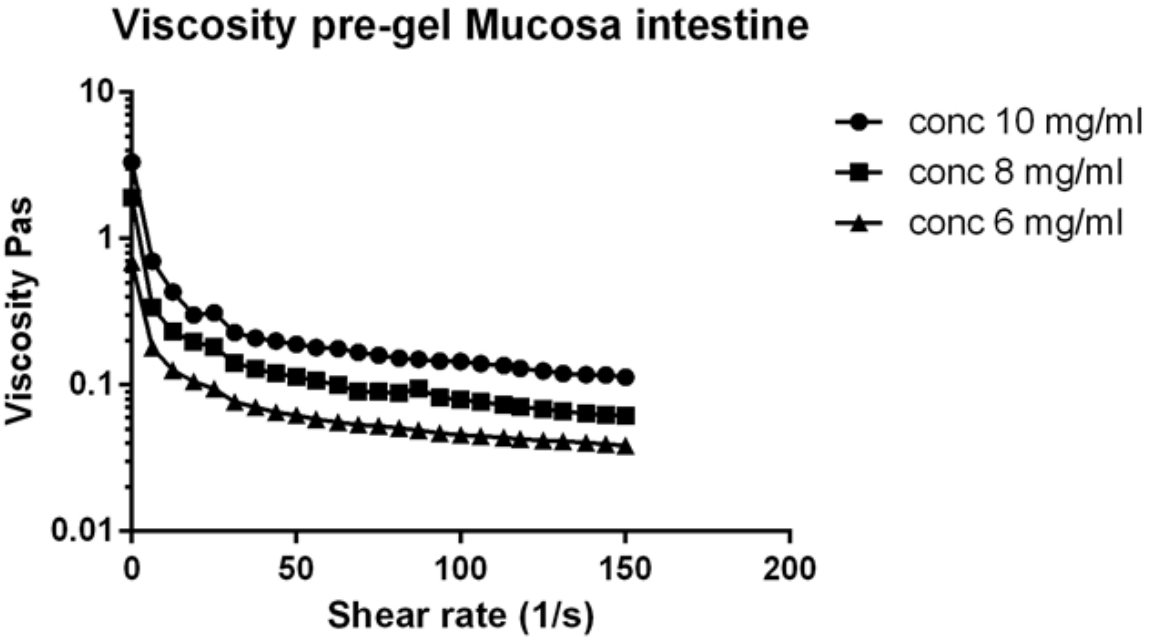

Figure 3.21. *Viscosity as function of shear rate for pre-gel mucosa intestine digested solution [Pas] for concentration of 10mg/ml, 8 mg/ml and 6 mg/ml obtained from DET decellularization*

As shown from results in fig. 3.21 it was possible to change the viscosity of the pre-gel solution acting on the dilution using 1 x PBS. By lowering the concentration of the ECM powder present in the solution was possible to lower also the viscosity in order to make it more easily to pipettes and to handle.
Comparing all the pre-gel initial viscosities, as shown in fig. 3.22, it was possible to observe a significant difference in the pre-gel solution viscosities between all the different dilutions ($p<0.005$) and also between different derivation ($p<0.005$). In particular whole intestine gel appeared to be have an higher initial viscosity compared to mucosa intestine for the concentration of 8 mg/ml and 10 mg/ml. The increase of viscosity at higher ECM powder concentration can be explained by an increase in intermolecular forces.

Because the material is shear thinning, in fact the viscosity decreased with an increase in the shear rate, it will have a lower viscosity when experiencing higher shear rates, such as those that occur when passing through a catheter or needle during an injection.

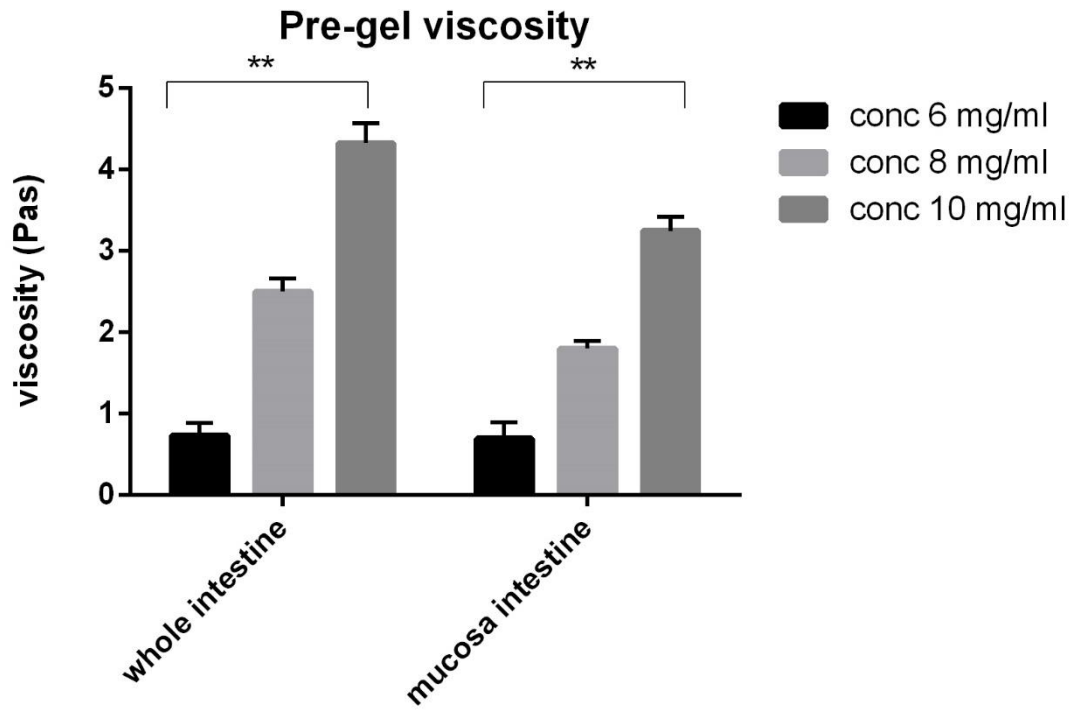

Figure 3.22. *Pre-gel viscosity comparison between whole intestine derived solution and mucosa intestine derived solution obtained with DET protocol for different dilution 10 mg/ml, 8 mg/ml and 6mg/ml [Pas]*

Again from SDS protocol viscosity results showed that also in this case the solution appear as shear thinning. The initial viscosity of the solution emerge also to be markedly lower than the one obtained from the DET protocol whole intestine powder for the same concentration.

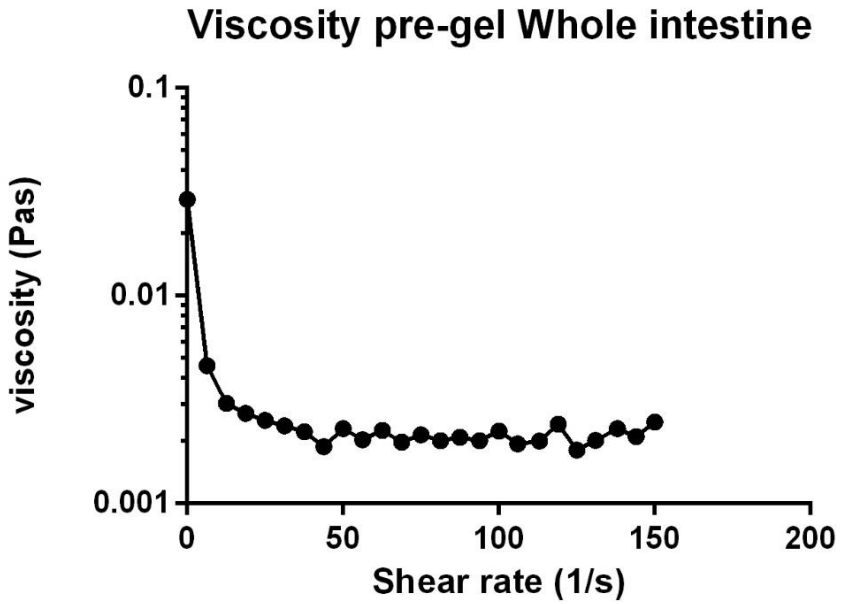

Figure 3.23. *Viscosity as function of shear rate for pre-gel whole intestine digested solution [Pas] for concentration of 10mg/ml obtained from SDS decellularization.*

3.2.3 Oscillatory test results

Elastic modulus (G') and viscous modulus (G") were measured at a shear stress of 0.5 Pa at frequency of 1 Hz. Since the temperature was imposed to be to 37 °C and the duration of the test was quite long, 1 hour, the test could cause evaporation of the samples, which could potentially effect the results. To minimize evaporation a plastic cap to be placed around the rheometer was designed. A wet tissue was then placed around the cap to provide wetness to the samples during the tests. From the graphs (fig 3.24, 3.25) it was no precisely possible to define a gelation point since it is not clear when the two curves cross each other.

For both whole intestine and (sub)mucosa samples it was possible to observe how elastic modulus reached higher values than viscous modulus meaning that gelation had occurred since the gel behave more like a solid than a liquid. Apart from whole intestine conc. 6mg/ml. In this case low values of storage modulus were observed.

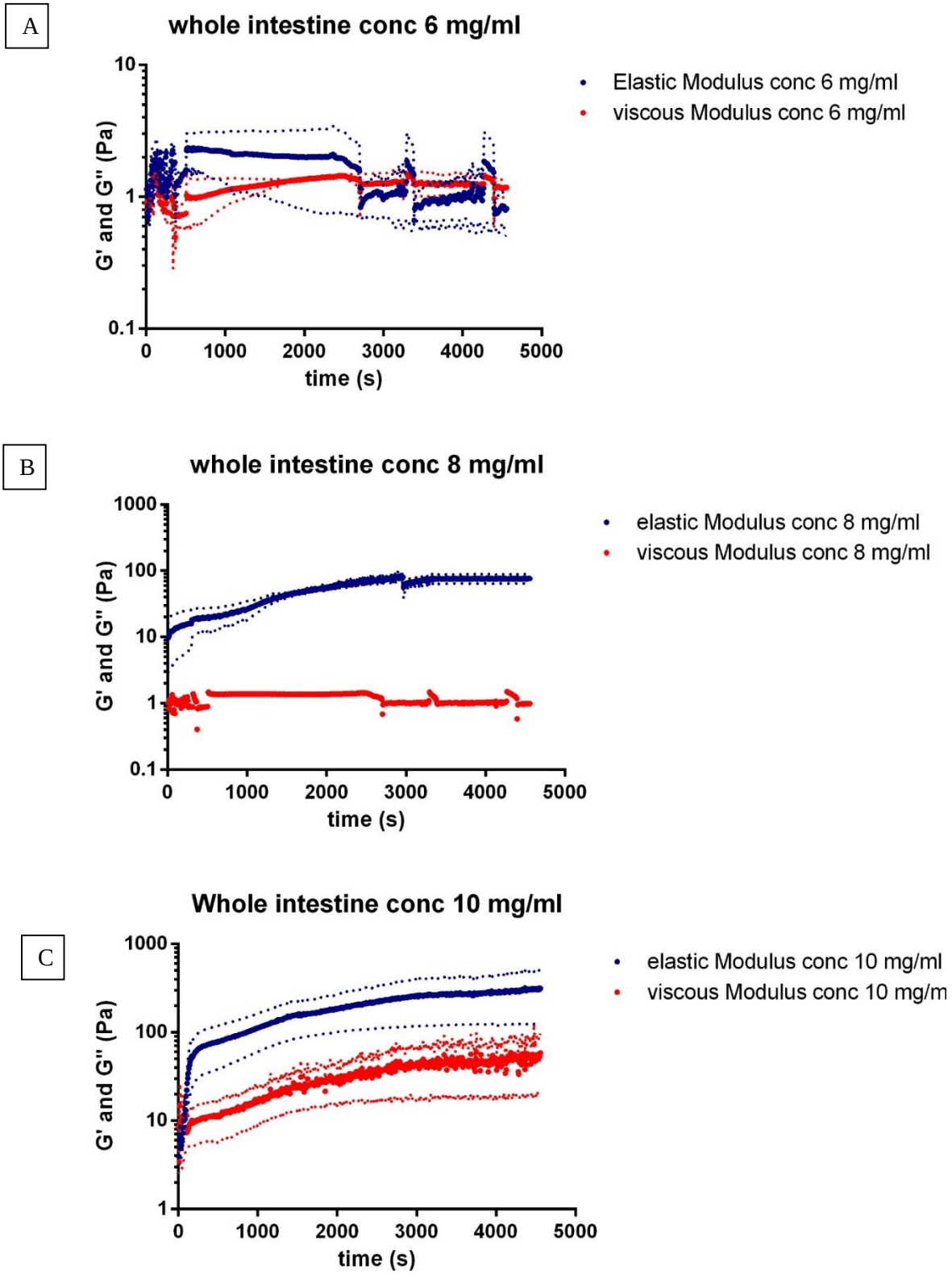

Figure 3.24. *Storage modulus(G') and loss modulus (G'') in function of time[s] for different concentration of ECM powder derived hydrogel obtained from whole intestine piglet decellularized with DET protocol. A) concentration of 6 mg/ml, B) concentration of 8 mg/ml and C) concentration of 10 mg/ml. Results are presented as a mean value with standard deviation.*

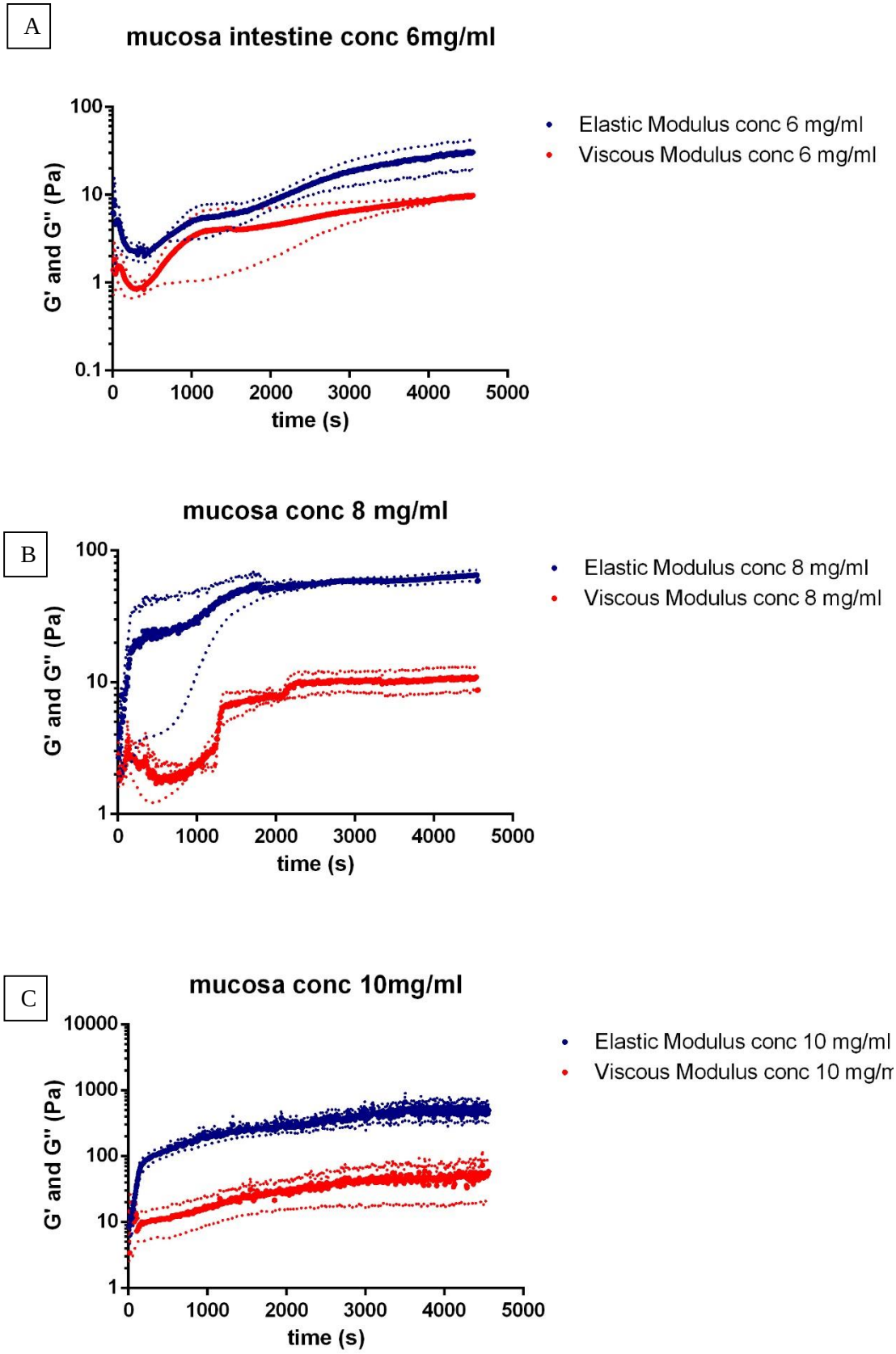

Figure 3.25. *Storage modulus(G') and loss modulus (G'') in function of time[s] for different concentration of ECM powder derived hydrogel obtained from mucosa intestine piglet decellularized with DET protocol. A) concentration of 6 mg/ml, B) concentration of 8 mg/ml and C) concentration of 10 mg/ml. Results are resented as a mean value with standard deviation.*

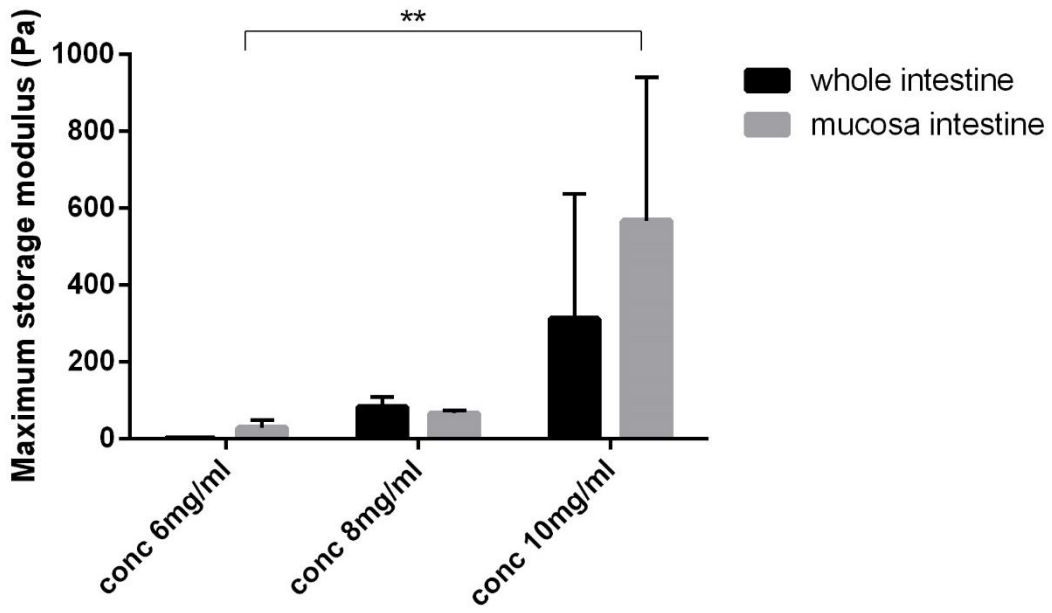

Figure 3.26. *Comparison of maximum storage modulus (G') of the hydrogel obtained from whole itnestine and (sub)mucosa ECM powder from DET protocl at different concentration. Results are presented as mean values with standard deviation.*

Maximum storage modulus was measured at the reached plateau and it was possible to observe that rheological properties of the ECM hydrogel appeared as concentration dependent. In particular best mechanical properties were obtained with the highest concentration of ECM powder. In fact significant difference ($p<0.05$) is observed between the different dilution while no statistical difference ($p>0.05$) is observed for the different derivation from whole intestine or mucosa intestine.

Results present a lot of variability especially for the concentration of 10 mg/ml, this suggest that more oscillatory test must be carried on. In order to improve the quality of result one possibility is to try to use mineral oil to avoid the evaporation of samples and also to change frequency and stress imposed.

Regarding to SDS protocol from result, fig. 3.27, gel appear to have no mechanical properties, at least not comparable with the one observed from the previous gel. This can be due to the fact that SDS protocol is more aggressive in term of protein denaturation from DET protocol, or maybe due to the fact that the solvent was not correctly removed from the tissue and so could cause the impossibility to gel.

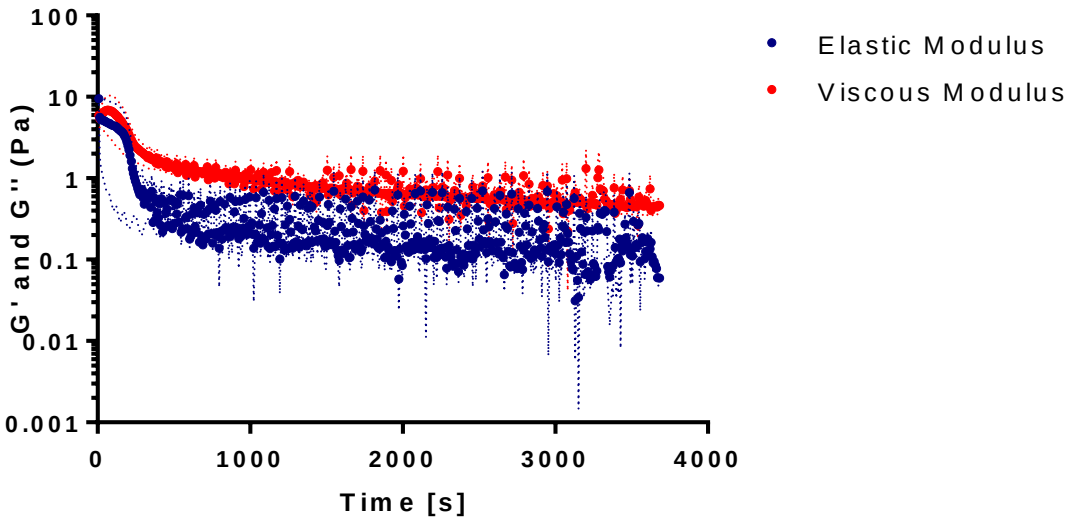

Figure 3.27. *Storage modulus(G') and loss modulus (G'') in function of time[s] for hydrogel obtained with ECM powder derived from whole intestine piglet decellularized with SDS protocol for concentration of 10 mg/ml. Results are presented as mean values with standard deviation.*

3.3 Citocompatibility of the hydrogel

Reagents used to prepare the hydrogel were sterilized by autoclaving after leaving the pepsin act for three day. It was observed that if the samples were put in the autoclave before the third day the digestion was suppressed because the action of pepsin was inhibited by low temperature.
The gel was then neutralized using sterile reagents and cell were seeded in different conditions. Cell viability was measured using MTT assay which represents the active mitochondrial enzymes present in a cell capable of reducing MTT. In this study the viability test was measured at 24 h and at 120 h after cell seeding.

Citocompatibility was tested using mucosa derived gel one since from a macroscopically point of view it appeared to be the more stable and also considering that its pre-gel viscosity was lower compared to the whole intestine derived hydrogel making it easier to handle.
The concentration which was chosen was 8 mg/ml because the initial viscosity of 10 mg/ml was considered too high and the gel was difficult to pipette.
Mouse embryonic fibroblast 3T3-J2 cells were chosen as a preliminary test before intestinal organoids for the citocompatibility because they were easily available and also because they

are the most common cells present in the connective tissue. Five different measurements were taken for each condition.

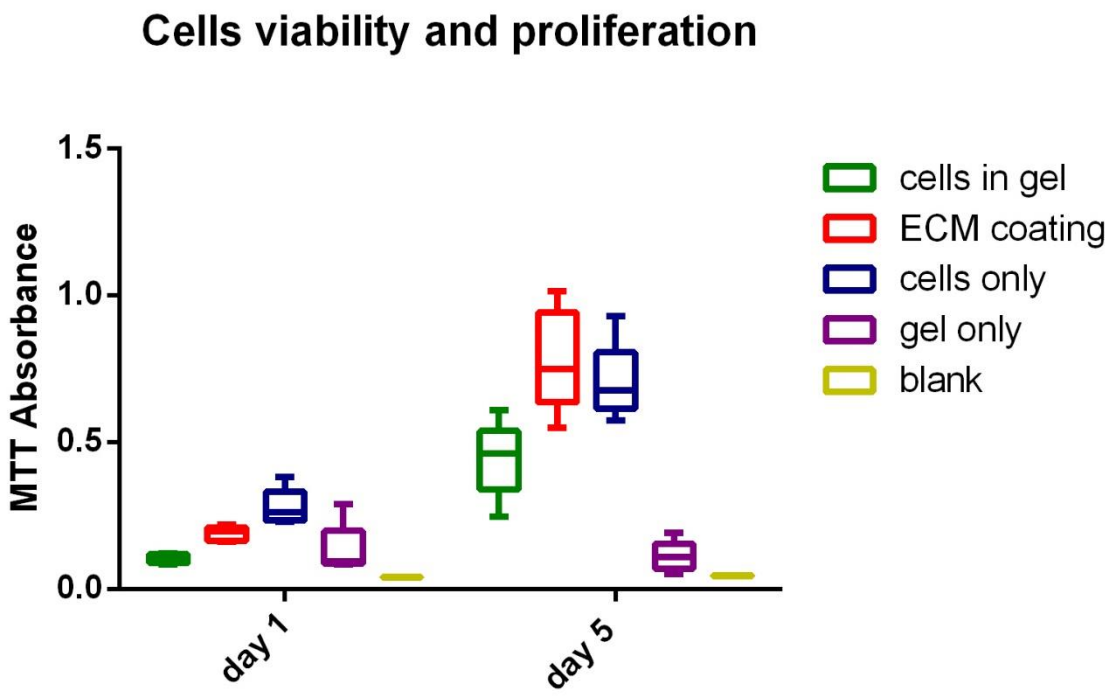

Figure 3.28. *Results of MTT assay tested for gel alone, TCP alone, ECM hydrogel coating and cells in the ECM hydrogel at day one and day 5.*

Table 3.4. *Statistical significance ($p<0.05$) between the different condition at day 1 and 5*

Condition	Day 1 v 5
Cells in gel	****
ECM coating	****
Cells only	****

Table 3.5. *Statistical significance ($p<0.05$) between the different conditions tested at day 5*

Condition	Cells in gel	ECM coating	Cells only
Cells in gel	/	****	****
ECM coating	/	/	ns

Results are presented in fig.3.28 and summarized in tab.3.4 and 3.5. It was possible to observe that there was a significant difference between day one and day 5 ($p<0.05$) between all the different condition tested.

The ability of the hydrogel to support viability and proliferation in ECM coating and petri dish alone shown that these samples evaluated exhibited comparable biocompatibility, in fact no statistical difference is observed (>0.05) from the results presented.
However cells in the gel appear to be less proliferating compared to ECM coating and the cells alone ($p<0.05$), but still they do increase the absorbance compared to day 1.

MTT method has been widely used as a suitable biocompatibility assay for in vitro evaluation of biomaterials.[23] Usually, cytotoxicity tests using cell cultures have been accepted as the first step in identifying active compounds and for biosafety testing. As a consequence, from MTT results it can be inferred that the hydrogel matrices produced in the present research showed potential to be tested in vivo assays and didn't present any evident element of toxicity for the tested cells.
In the case of cells in the gel they did not proliferate as well as in the ECM coating or cells alone. It can be due to the assay which has been used. In fact one possibility of this discrepancy is that probably cells were trapped into the gel and so the colour from the dye couldn't be released properly, results is that the spectrometer couldn't read it correctly.

3.4 Organoids Test results

Organoids culture was preliminary tested in order to observe the potential of the ECM derived hydrogel using a 8mg/ml concentration gel derived from mucosa intestine considering this to be the best candidate taking into account the same consideration previously reported. Two different gel were tested.

BME, equivalent of Matrigel, was used as comparison since until now from Sato's original description of long term murine small intestinal epithelial culture most of studies have all employed it as an extracellular matrix for three-dimensional cell suspension.

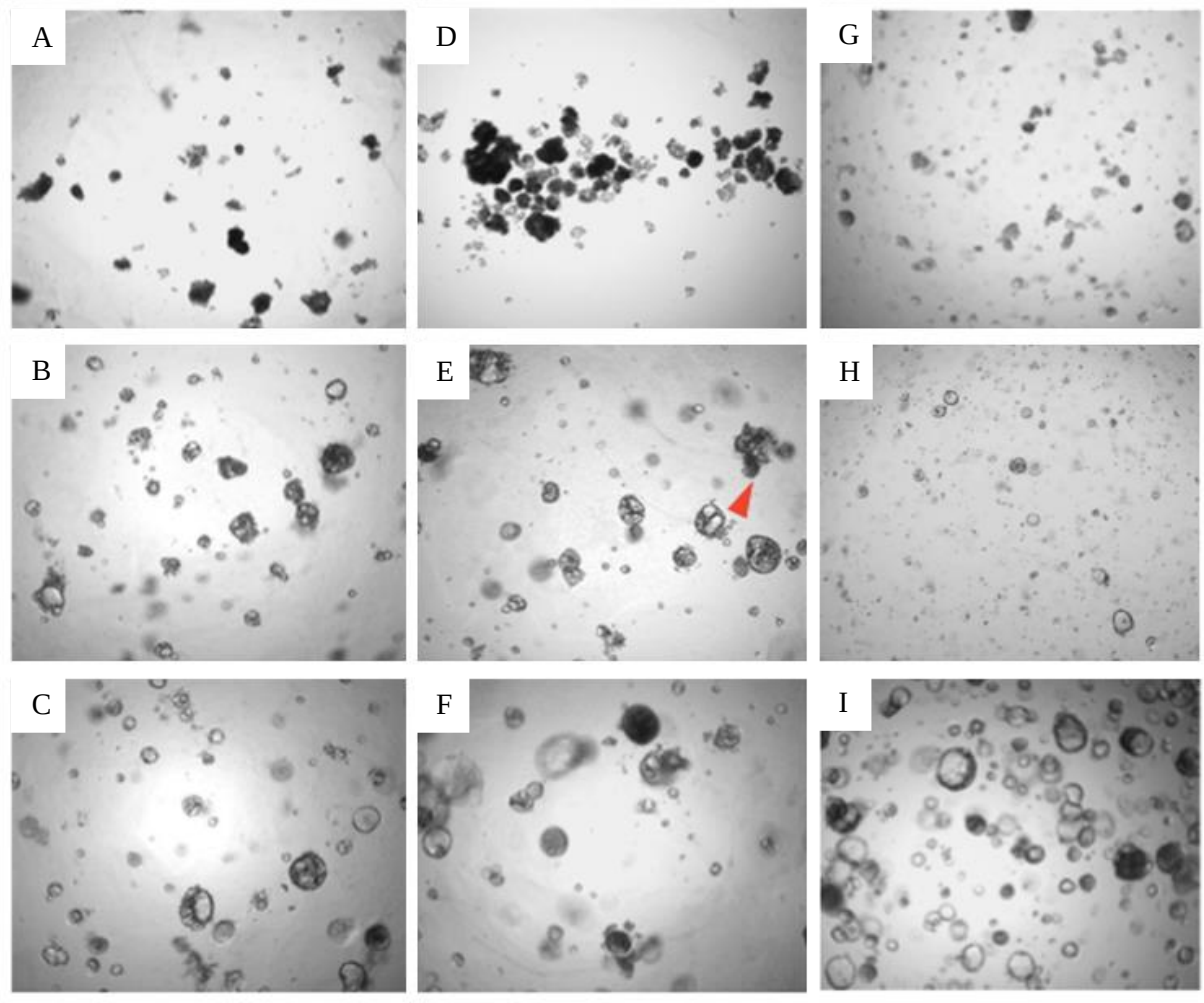

Figure 3.29. *Images taken on 5x objective on inverted microscope of Organoids cultured in gel 1 A) at day 0, B) at day 3 and C) at day 6. Images of Organoids cultured in gel 2 D) at day 0, E) day 3 and F) day 6. Matrigel is used as comparison in order to qualitatively asses the difference in morphology and proliferation of organoids F) at day 0, G) day 3 and H) day 6. Images are taken in clumps of gel which were not deteriorating.*

Small intestinal organoids were used after trypsinization and seeding cell clumps. From the results of the preliminary test it was observed that quality of gel in culture deteriorated and detached from the base of the tissue culture plate soon after seeding droplets in both cases. As a result, by Day 3 organoids were seen to attach to the base of the tissue culture plate and lose their 3D morphology (red arrowhead) or desegregate (fig 3.29 E).

Images were taken at Day 0 (one hour after embedding in the gel), Day 3 and Day 6. Best images were taken of surviving organoids founded in clumps of the gel that were floating in the media. In this case organoids seems to maintain their characteristic morphology similar to one cultured into Matrigel (Fig 3.29).

In fig. 3.30 is shown the difference in results after day 3 of another batch of sample. It was observed that there was a clear difference between behave of the intestinal organoids in the different area of the gel. Yellow arrow indicate an healthy organoids while red arrow indicate a desegregated one, clear difference in morphology is observed. Healthy organoids preserve 3D structure while desegregated one are spread and attached to the bottom of TCP (red arrow fig. 3.30).

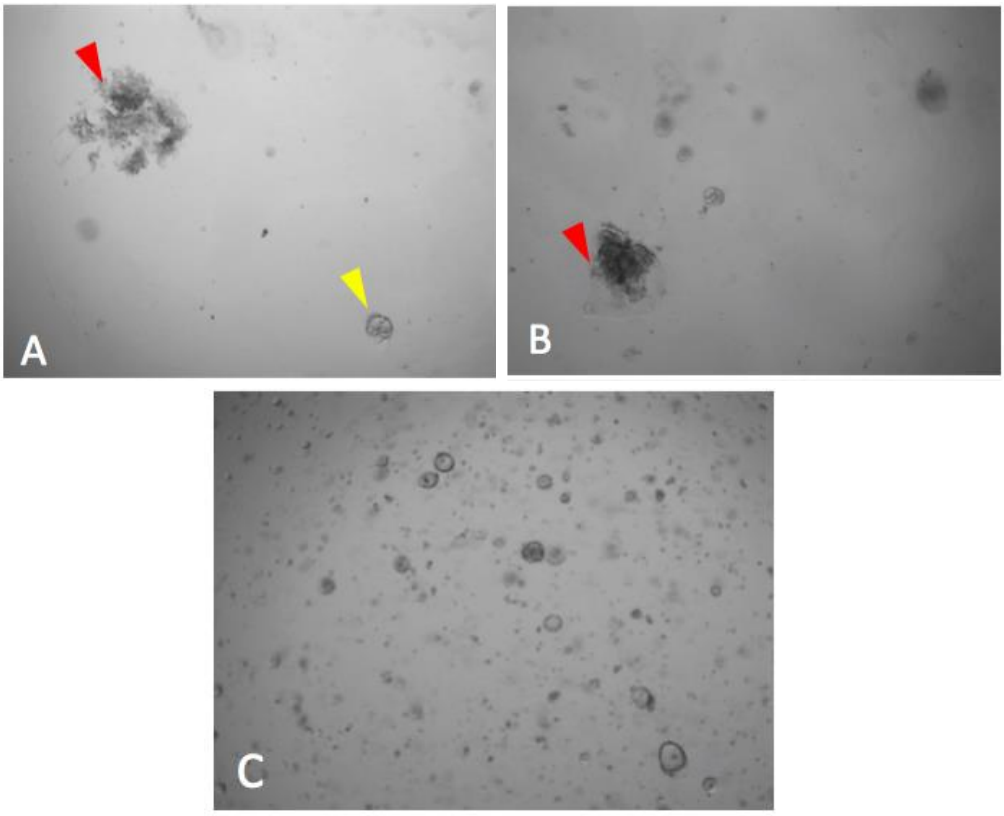

Figure 3.30. *Images taken on 5x objective on inverted microscope of seeded organoids into A) gel 1, B) gel 2 and C) Matrigel at day three. Red arrow indicate a desegregated organoids while yellow arrow indicate an healthy organoids which conserve its 3D structure.*

Taking into account the preliminary results it is believed that there can be promising results for the grow of intestine organoids in the porcine mucosa derived hydrogel but still a lot of work must be done in order to optimize the durability of the gel.
One approach that can be followed to improve gel durability is to combine the benefits of natural and synthetic materials.
Unlike synthetic or single component protein hydrogels, ECM derived materials retain a complex mixture of tissue specific biochemical cues, including both proteins and polysaccharide, but the result of the self-assembly is a soft hydrogel with low storage modulus, which may have the inconvenient of not allowing for in vitro 3D culture since cells can rapidly migrate through the soft hydrogel or it can easily deteriorate.
Altering the properties of protein-based hydrogel using synthetic hybrid hydrogel has been already studied by Christman et all., where it was possible to improve the stiffness of the gel by doping it with synthetic polymers. All the properties of collagen hydrogels were altered by cross-linking the ECM-based hydrogel with multi-armed poly(ethylene glycol) (PEG) stars containing activated esters on the termini that reacted with amine residues on the protein[50]. PEG was considered an ideal candidate to add to protein hydrogels because it is biocompatible and inert, and therefore the biochemical cues in the hydrogel remain the same. [51]

It is also important that after having improved the mechanical stability of the gel some biochemical analysis on the organoids structure must be carried on. As reported in previous work were in order to determine the difference in gene expression from Matrigel cultured organoids with Collagen 1 cultured organoids some gene were determined. Quantitative real-time polymerase chain reaction (qPCR) was performed to determine expansion of the transgenic eGFP-DNA and to determine the mRNA gene expression profile. [52]

Conclusions

Starting from the consideration that each tissue present a distinct composition of ECM in which the appropriate signalling molecules and structural components are present to allow for cell growth, differentiation and synergized tissue function[53], it is reasonable to assume that decellularization of site-specific tissue would provide the optimal inductive template for tissue engineering in its respective anatomic location. For this reason the optimisation of a protocol for the decellularization of the piglet whole intestine and (sub)mucosa intestine was successfully developed. Results showed that one cycle of DET protocol and one day of SDS protocol were considered sufficient for the removal of the nuclei content from the tissue. H&E, DAPI staining and DNA quantification assay were used to assess the efficacy of the decellularization.

This newly developed and optimised protocol is now validated for further uses in the future. Immunostaining analysis for major histocompatibility complex II (MHC-II), which are molecules that are present at the surface of the cells and that elicit a response of the immune system, could be performed to show the absence in the decellularized tissues and further confirm the quality of the obtained protocol.

In order to confirm presence of some important ECM component in the obtained decellularized tissue such as Collagen, Elastin and GAGs were adopted Picro Serius Red, purple EVG and Alcian Blue stainings. More investigation must be carried on to quantify the conservation of growth factor in the gel after the digestion in HCl and pepsin. Grow factors in fact are immobilized by Sulfated glycoaminoglycans (GAGs), which content present a small decrease as shown by the results of the Blyscan GAG assay, so there may be the possibility to have tissue specific grow factor in the decellularized tissue as reported from study of K. Christman's group; ECM derived hydrogels (made from pig decullarised pericardium) exhibited an enhanced retention and delivery of angiogenic growth factors (bFGF) compared to collagen gels. In a tissue engineering approach GAGs could also be potentially helpful in the case in which some specific grow factors absent in the gel would be required to improve cell culture for instance.

The obtainment of an hydrogel from ECM was successfully reached from both whole intestine and (sub)mucosa intestine derived from DET protocol while SDS protocol led to a poor quality gel which lacked mechanical properties. The reason of this have not been elucidated until now also if some hypothesis have been proposed. SDS treatment could in fact be more aggressive in destruction of the ECM component, or there could be the possibility that the solvent was not completely removed after the treatment and could interfere in the process of self-assemble of the peptides.

Some improvement can be done in the phase of the gel fabrication starting from a way to limit the loss of lyophilised powder during the milling which can be easily improved by the processing of a larger starting material. In fact the quantity of lost tissue is mostly independent on the quantity of initial lyophilized. An additionally stage could also be added to the adopted protocol, the gels in fact were always used directly after neutralisation, however for practical reason having the lyophilized already neutralized before each use could be desirable.
The addition of a second freeze drying step after neutralisation would turn the digested solutions back into ECM powder that would be in that manner already neutralised. This could lead to a more precise dilution considering that it easier to weight a certain amount of powder and dilute it in 1XPBS rather than pipette few millimetres of digested solution which is very viscous and dilute it in PBS, batch-to bath variation could be reduced.

Rheological properties of the gel were observed to be concentration dependent which lead to the decision to consider the 8mg/ml mucosa intestine derived hydrogel the best candidate in term of balance between storage modulus and pre-gel viscosity. In fact also if 10 mg/ml concentration gel was observed to have the highest storage modulus and best consistency from a macroscopic point of view, its pre-gel viscosity was considered too high for an easy utilization and for pipetting. Moreover it was observed that results obtained from oscillatory test presented a lot of variability meaning that more experiment must be carried on to best investigate the stiffness of the obtained product. Atomic Force Microscopy, AFM, may be one useful tool to investigate elastic modulus. In fact recent studies elucidated how matrix morphology and matrix mechanical properties can have pronounced effects on several cellular processes including adhesion, spreading, migration, and proliferation. Therefore, changes in ECM density and storage modulus have the potential to produce profound effects on the outgrowth and self-organization of encapsulated three-dimensional cultures. For example, changes in collagen matrix density have been shown to influence the density, cross-sectional area, and path-finding abilities of endothelial cell-derived vessels in vitro and in vivo.
Moreover recent studies has shown how contractile behaviour of organoids occurred only in a narrow range of matrix density with storage moduli of near 30 Pa.[54] This suggested that matrix density acts a ‘‘permissive switch’’ that enables contractions to occur. As peristaltic contractility is a crucial requirement for normal digestive tract function, this achievement of reproducible organoid contraction marks a pivotal advancement towards engineering physiologically functional replacement tissue constructs that must be taken into account.

Test one citocompatibility showed how no evident toxic component were left in in the hydrogel after the production cycle meaning that it can be potentially adopted in the field of tissue engineering. Finally organoids test elucidated some important potential in the use of the hydrogel for three-dimensional (3D) cell culture systems but also pointed out the need to find

a solution in term of durability and stability of the gel. The possibility of creating a hybrid hydrogel could be one of the solution in order to overcome the mechanical issues encountered while maintaining the tissue specific nature. It has been in fact previously reported how an hybrid hydrogel obtained from PEG incorporation into ECM-based hydrogels can expand mechanical properties, thereby opening up new possibilities for in vitro and in vivo applications.[51]

In conclusion, a successful protocol was established to prepare an ECM derived hydrogel from piglet intestinal tissues that has several promising potential applications, and which can be in the future a useful tool in tissue engineering as an improved culture media to grow intestinal cells with the capability to eliminate the need for Matrigel. This is a critical step towards the production of neo-mucosa using good manufacturing practices for clinical application in the future.

Bibliography

1. Excellence, Q. A. & Care, H. Agency for Healthcare Research and Quality Advancing Excellence in Health Care •. (2010).

2. Katari, R. S., Peloso, A. & Orlando, G. Tissue engineering. *Adv. Surg.* **48,** 137–54 (2014).

3. Mccoy, C. P. *et al.* Optimization of singlet oxygen production from photosensitizer-incorporated , medically relevant hydrogels. 1–7 (2015). doi:10.1002/jbm.b.33562

4. Chapekar, M. S. Tissue Engineering : Challenges and Opportunities. **20899,** 617–620 (2000).

5. O'Neill, J. D., Freytes, D. O., Anandappa, A. J., Oliver, J. a. & Vunjak-Novakovic, G. V. The regulation of growth and metabolism of kidney stem cells with regional specificity using extracellular matrix derived from kidney. *Biomaterials* **34,** 9830–9841 (2013).

6. El-Sherbiny, I. & Yacoub, M. Hydrogel scaffolds for tissue engineering: Progress and challenges. *Glob. Cardiol. Sci. Pract.* **2013,** 316–42 (2013).

7. Conconi, M. T. *et al.* Tracheal matrices, obtained by a detergent-enzymatic method, support in vitro the adhesion of chondrocytes and tracheal epithelial cells. *Transpl. Int.* **18,** 727–734 (2005).

8. Totonelli, G. *et al.* A rat decellularized small bowel scaffold that preserves villus-crypt architecture for intestinal regeneration. *Biomaterials* **33,** 3401–3410 (2012).

9. Baptista, P. M. *et al.* The use of whole organ decellularization for the generation of a vascularized liver organoid. *Hepatology* **53,** 604–617 (2011).

10. Järveläinen, H. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol Rev.* **61,** 198–223 (2009).

11. Frantz, C., Stewart, K. M. & Weaver, V. M. The extracellular matrix at a glance. *J. Cell Sci.* **123,** 4195–4200 (2010).

12. Rozario, T. & DeSimone, D. W. The extracellular matrix in development and

morphogenesis: A dynamic view. *Dev. Biol.* **341,** 126–140 (2010).

13. Wilusz, R. E., Sanchez-Adams, J. & Guilak, F. The structure and function of the pericellular matrix of articular cartilage. *Matrix Biol.* **39,** 25–32 (2014).

14. Sambasivarao, S. V. NIH Public Access. **18,** 1199–1216 (2013).

15. Colognato, H. & Yurchenco, P. D. A PEER REVIEWED FORUM Form and Function : The Laminin Family of Heterotrimers. **234,** 213–234 (2000).

16. Miner, J. H. Laminins and Their Roles in Mammals. **356,** 349–356 (2008).

17. Bosman, F. T. & Stamenkovic, I. Functional structure and composition of the extracellular matrix. *J. Pathol.* **200,** 423–428 (2003).

18. Han, D. & Gouma, P.-I. Electrospun bioscaffolds that mimic the topology of extracellular matrix. *Nanomedicine Nanotechnology, Biol. Med.* **2,** 37–41 (2006).

19. Geckil, H., Xu, F., Zhang, X., Moon, S. & Demirci, U. Engineering hydrogels as extracellular matrix mimics. *Nanomedicine (Lond).* **5,** 469–484 (2010).

20. Vasconcelos, A. C. *et al.* Foreign Body Response to Subcutaneous Implants in Diabetic Rats. **9,** (2014).

21. Wulkersdorfer, B. *et al.* Growth Factors Adsorbed on Polyglycolic Acid Mesh Augment Growth of Bioengineered Intestinal Neomucosa. *J. Surg. Res.* **169,** 169–178 (2011).

22. Manuscript, A. NIH Public Access. **32,** 3233–3243 (2012).

23. Medical, T. A Simple In Vito Cytotoxicity Test Using the MTT (3- (4 , 5) Tetrazolium Bromide) Colorimetric Assay : Analysis of Eugenol Toxicity on Dental Pulp Cells (RPC-C2A). (1989).

24. Badylak, S. F., Taylor, D. & Uygun, K. Whole-Organ Tissue Engineering : Decellularization and Recellularization of Matrix Scaffolds. doi:10.1146/annurev-bioeng-071910-124743

25. Cristina, L., Nazareno, F. De, Laurindo, C. A. H., Affonso, F. D. & Moreno, A. N. Effects of cryopreservation and / or decellularization on extracellular matrix of porcine valves. **26,** 490–496 (2011).

26. Stock, U. A. & Hoerstrup, S. P. Tissue engineering of heart valves using decellularized xenogeneic or polymeric starter matrices. 1505–1512 (2007). doi:10.1098/rstb.2007.2131

27. Peppas, B. N. A., Hilt, J. Z., Khademhosseini, A. & Langer, R. Hydrogels in Biology and Medicine : From Molecular Principles to Bionanotechnology **. 1345–1360 (2006). doi:10.1002/adma.200501612

28. Hoffman, A. S. Hydrogels for biomedical applications ☆. *Adv. Drug Deliv. Rev.* **64,** 18–23 (2012).

29. Peppas, N. a., Hilt, J. Z., Khademhosseini, A. & Langer, R. Hydrogels in biology and medicine: From molecular principles to bionanotechnology. *Adv. Mater.* **18,** 1345–1360 (2006).

30. Zhu, J. & Marchant, R. E. Design properties of hydrogel tissue-engineering scaffolds. *Expert Rev. Med. Devices* **8,** 607–626 (2011).

31. Manuscript, A. NIH Public Access. **103,** 655–663 (2010).

32. Monteiro, N., Martins, A., Reis, R. L. & Neves, N. M. Nanoparticle-based bioactive agent release systems for bone and cartilage tissue engineering. *Regen. Ther.* **1,** 109–118 (2015).

33. Jin, R. & Dijkstra, P. J. Hydrogels for Tissue Engineering. *Chem. Rev.* **101,** 1869–1879 (2001).

34. Hughes, C. *et al.* Mass Spectrometry – based Proteomic Analysis of the Matrix Microenvironment in Pluripotent Stem Cell Culture * □. 1924–1936 (2012). doi:10.1074/mcp.M112.020057

35. Barker, N. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat. Rev. Mol. Cell Biol.* **15,** 19–33 (2013).

36. Byrne, T. a. *et al.* Growth Hormone, Glutamine, and an Optimal Diet Reduces Parenteral Nutrition in Patients With Short Bowel Syndrome. *Ann. Surg.* **242,** 655–661 (2005).

37. Tzakis, a G. *et al.* Clinical intestinal transplantation: focus on complications. *Transplant. Proc.* **24,** 1238–1240 (1992).

38. Summary, R. Organogenesis in a dish: Modeling development and disease using organoid technologies. doi:10.1126/science.1247125

39. Sato, T. & Clevers, H. Growing Self-Organizing Mini-Guts from a Single Intestinal Stem Cell: Mechanism and Applications. **340,** 1190–1195 (2013).

40. Sellaro, T. L. *et al.* Maintenance of Human Hepatocyte Function In Vitro by Liver-Derived Extracellular Matrix Gels. **16,** (2010).

41. Crapo, P. M., Gilbert, T. W. & Badylak, S. F. Biomaterials An overview of tissue and whole organ decellularization processes. *Biomaterials* **32,** 3233–3243 (2011).

42. Gilbert, T., Sellaro, T. & Badylak, S. Decellularization of tissues and organs. *Biomaterials* **27,** 3675–3683 (2006).

43. Cebotari, S. *et al.* Detergent Decellularization of Heart Valves for Tissue Engineering : Toxicological Effects of Residual Detergents on Human Endothelial Cells. **34,** 206–210 (2010).

44. Ulijn, R. & Woolfson, D. Peptide- and protein-based materials themed issue and other applications w. (2010). doi:10.1039/b919449p

45. Winter, H. H. & Chambon, F. Analysis of Linear Viscoelasticity of a Crosslinking Polymer at the Gel Point. *Journal of Rheology* 367–382 (1986).

46. Manuscript, A. NIH Public Access. **30,** 1482–1491 (2010).

47. Wood, G. C. & Keech, M. K. The Formation of Fibrils from Collagen Solutions 2. 598–605 (1960).

48. Achilli, M. & Mantovani, D. Tailoring Mechanical Properties of Collagen-Based Scaffolds for Vascular Tissue Engineering: The Effects of pH, Temperature and Ionic Strength on Gelation. 664–680 (2010). doi:10.3390/polym2040664

49. Stuart, K., Panitch, A., Engineering, B., Drive, M. J. & Lafayette, W. Kate Stuart, Alyssa Panitch. **89,** (2008).

50. Browning, M. B., Russell, B., Rivera, J., Ho, M. & Cosgri, E. M. Bioactive Hydrogels with Enhanced Initial and Sustained Cell Interactions. (2013).

51. Grover, G. N., Rao, N. & Christman, P. K. L. NIH Public Access. **25,** 1–23 (2015).

52. Jabaji, Z. *et al.* Use of Collagen Gel as an Alternative Extracellular Matrix for the In Vitro and In Vivo Growth of Murine Small Intestinal Epithelium. **19,** 961–969 (2013).

53. Keane, T. J. *et al.* Preparation and characterization of a biologic scaffold and hydrogel derived from colonic mucosa. **1247842,** 1–16 (2015).

54. Heilshorn, P. S. C. Integrative Biology. **6,** (2014).

55. Sell, S. *et al.* Extracellular matrix regenerated : tissue engineering via electrospun biomimetic nanofibers. **1360,** 1349–1360 (2007).

56. Tibbitt, M. W. & Anseth, K. S. Hydrogels as Extracellular Matrix Mimics for 3D Cell Culture. **103,** 655–663 (2009).

Ringraziamenti

Alla fine di questa tesi e di questi splendidi anni di Università desidero ringraziare tutti coloro che mi hanno aiutato in questo percorso con suggerimenti, critiche ed osservazioni. A loro va la mia gratitudine. Ringrazio anzitutto il professor Nicola Elvassore per avermi dato la possibilità di crescere e imparare. Il Professor Paolo De Coppi per avermi accolta nel suo laboratorio facendomi sempre sentire a mio agio. Ringrazio Claire per avermi indirizzata con gentilezza e umiltà in molte scelte lasciandomi però libera di sbagliare e riprovare, insieme a tutti i ragazzi del laboratorio di Londra che mi hanno sempre dato consigli e con i quali ho vissuti momenti bellissimi.

Un doveroso e sincero ringraziamento va a mia madre, mio padre e i miei fratelli per la loro inconfondibile presenza. Mi avete sempre fatto sentire il vostro sostegno forte e chiaro e non avete mai smesso di credere in me. Non ce l'avrei mai fatta senza di voi.

Ringrazio la mia famiglia padovana e i miei amici Tiz, Matte, Giulio, Gio, Gallo, Albe, Nik$_S$, Sacha, Cava, Manu e tutti gli altri, le parole non basteranno mai a dimostrare la mia immensa gratitudine. Battiato diceva "Il tempo cambia molte cose nella vita il senso le amicizie le opinioni che voglia di cambiare che c'è in me..." tutto vero ma quando cerco di volgere lo sguardo avanti per capire cosa mi aspetta non riesco a non vedervi vicino a me. Siete la mia certezza.

Voglio poi ringraziare i miei compagni e amici ingegneri con i quali ho condiviso l'entusiasmo, ma anche la fatiche di questo percorso. Ogni momento è stato vero e bello con voi al mio fianco. E non posso tralasciare il mio "gruppo studio", senza il quale non avrei mai scritto questa tesi. Lo studio è studio ma le pause sono vita.

Vorrei che questi ringraziamenti siano un punto di arrivo ma anche un inizio, perché non si deve mai smettere di andare avanti a crescere e imparare e spero che in qualsiasi futuro ci sia in serbo per me voi sarete tutti ancora presenti.