Design and construction of a decellularization cell

Design and construction of a decellularization cell that acts by perfusion technique

Mestrado **I**ntegrado em **B**ioengenharia

Inês Odila Pereira
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Para a minha e para a "força da natureza" da Maria,

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"O meu dia-a-dia começa a ser uma espécie de listagem de opções. Cada vez vou observando mais escolhas e apercebo-me que quero muitas delas..."

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Abstract

It is well known that the extracellular matrix components (ECM) are key modulators of cell behavior and thus, a major effort is being paid to understand how ECM is modulated by and modulates cellular activities such as migration, adhesion, survival and differentiation. This background excited our interest in creating an innovative matrix based in human ECM that would lead to a better understanding of how ECM modulates cellular activities during tissue regeneration or cancer cell invasion and metastasis.

This potential found in ECM led us to the designing and constructing of a decellularization apparatus – Decellularization Cell (DecCell) – dedicated to a successful removal of cells while maintaining the mechanical, chemical and structural properties of the ECM.

Our goal was accomplished since the DecCell is an innovative system, functional for different sizes and thickness of tissues, with an automatic control and providing a dynamic perfusion - a new concept of decellularization technique. In the near future, the efficiency of the DecCell will be tested by decellularization of human-derived matrices from colon fragments of cancer and non-cancer patients.

We believe that this research may lead to a crucial step for bioengineering fields and oncobiology approaches.

Keywords: Extracellular matrix, construction, decellularization, design, perfusion technique

Resumo

Os componentes da matriz extracelular (MEC) modelam o comportamento das células e, desta forma, o modo como a MEC é modulada e modula as diversas actividades celulares tais como a migração, adesão, sobrevivência e diferenciação, tem sido explorado.

Este facto suscitou o nosso interesse em desenvolver uma matriz extracelular humana que nos levará a uma melhor compreensão do papel da MEC, quer em fenómenos celulares presentes na regeneração de tecidos, quer na invasão do cancro e metástases. Assim, criou-se como objectivo a concepção (*design*) e construição de uma máquina de descelularização – *"Decellularization Cell (DecCell)"* – dedicada à remoção de células de diferentes tecidos, mantendo as propriedades mecânicas, químicas e estruturais da matriz extracelular.

O objectivo a que nos propusemos foi alcançado, já que a DecCell se revelou um sistema inovador, por ser funcional para tecidos de diferentes tamanhos e espessuras, ter um controlo automatizado e por usar a técnica de perfusão – um novo conceito na aplicação dos agentes de descelularização.

Num futuro próximo, a eficiência da DecCell deverá ser testada através da descelularização de matrizes humanas provenientes de tecidos do cólon, cancerosos ou não. Acreditamos que este projecto poderá determinar um avanço extremamente importante em áreas como a bioengenharia e da oncobiologia.

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List of Abbreviations

3D - Three dimensional

CHAPS – 3-[(cholamidopropyl) dimethyl ammo-nio]-1-propanesulfonate

DAPI – 4', 6-diamidine-2-phenylindole

DDR1 – Discoidin domain receptor family number 1

DEM – Detergent-enzymatic method

DMA – Dynamic mechanical analysis

DMMB – Dimethylmethylene blue

DNA – Desoxyribonucleic acid

DNase – Deoxyribonuclease

ECM – Extracellular matrix

EDTA – Ethylene-diamine-tetra-acetic acid

EGF-like repeats – Epidermal growth factor-like repeats

EGFR – Epidermal growth factor receptor

ERK – Extracellular signal-regulated kinases

FGF – Fibroblast growth factor

GAGs – Glycosaminoglycans

LSM – Laser scanning microscopy

PAA – Peracetic acid

RGD – Arginine-glycine-aspartic acid

RNA – Ribonucleic acid

SDS – Sodium dodecyl sulfate

SEM – Scanning Electron Microscope

SLRPs – Small leucine-rich proteoglycans

SPARC – Secreted protein acidic and rich in cysteine

TGF-β – Transforming growth factor beta

TNF-α – Tumor necrosis factor-alpha

1. Introduction

A.

The Extracellular Matrix

For many years, extracellular matrix (ECM) was thought to serve only as a structural support for cells, required to maintain tissue architecture. Currently, is well known that ECM is a key modulator of cell behavior providing individual cells with architectural cues of time and space and modulating the bioavailability of many enzymes, peptides, growth and differentiation factors. Nowadays, a major effort is being paid to understand how the microenvironment that surrounds the cell is modulated by and modulates cellular activities. In this chapter emphasis will be given to the molecular composition and structural organization of major ECM components as well as to their role on the modulation of distinct cellular activities.

1.1. ECM composition and diversity

ECM is a complex molecular network that surrounds and supports cells, being composed, essentially, by: i) fibrous proteins that provide tensile strength and elasticity (e.g., collagens and elastins), ii) adhesive glycoproteins (e.g., fibronectin and laminin), and iii) proteoglycans that interact with other ECM components to provide a hydrated gel that resists to compressive forces (Varki A 2009). This molecular complex also arrests many molecules such as growth and differentiation factors, cytokines, and matrix-degrading enzymes (such as matrix metalloproteases) and their inhibitors. The distribution and organization of these molecules affect tissue function and may vary from tissue to tissue along adult life or during embryonic development (Anthony Atala 2010). While ECM is one of the major elements of bone and cartilaginous tissues, it is the minor element of brain and spinal cord (Egeblad and Werb, 2002). Some of the major ECM components, their biochemical properties and function, will be presented.

Collagen

Collagens consist of a family of fibrous and non-fibrous proteins synthesized by connective tissue resident cells, such as fibroblasts, chondroblasts, osteoblasts, and odontoblasts), and secreted into the ECM via exocytosis. Being the major component of skin and bone, collagen is the most abundant ECM element, constituting approximately 25% of the total mammalian protein mass (Bruce Alberts 2002). Collagen consists of a unique triple helical structure built by three intertwined polypeptide chains, which in turn are composed by multiple repetitions of Gly-X-Y motifs in which the amino acid residues in X- and Y- positions are frequently proline and hydroxyproline. The collagen family can be classified into different subfamilies according to their supramolecular assembly. Hence, different collagen types are necessary to confer distinct biological features to the various types of connective tissues present in the body. The major ones are: a) Fibrilar collagens which form fibrils that are associated in fibers such as collagen type I, the major component of tendons, ligaments, and bones, collagentype II, which represents more than 50% of the protein in cartilage, and collagentype III that strengthens the arteries or intestine walls; b) Fibril-associated collagens which link distinct collagen fibrils, such as collagen type VI, IX or XII; c) Networking collagens such as collagen type IV, a highly specialized form found in

basement membranes underlying epithelial and endothelial cells (Friess 1998); and d) Cell membrane-associated collagens, such as collagen XVII and XVIII.

Elastin

Elastin, another structural component, is a hydrophobic protein that forms extracellular extensible fibers, being responsible for stretch ability and the resilience of tissues (Debelle and Tamburro 1999). Elastin serves an important function in as a medium for pressure wave propagation to help blood flow and is particularly abundant in large elastic blood vessels such as the aorta. Elastin is also very important in the lungs and elastic cartilage. It is present in all vertebrates above the jawless fish.

Fibronectin

Fibronectin is a dimeric glycoprotein present at the cell surface and deposited as highly insoluble fibrils in the ECM or blood. This protein can play its role in a variety of biological processes, due to its ability to interact with cells and many ligands such as heparin, fibrin, collagen, DNA and immunoglobin (Michael J. Yaszemski 2004). The arginine-glycine-aspartic acid (RGD) cell adhesion sequence was discovered in fibronectin for Pierchbacher and Ruoslahti in 1984. This finding and the subsequent discovery of integrins ascell surface receptors that recognize RGD sequences within various proteins, gave to RGD a central role in cell adhesion biology (Ruoslahti 1996). Therefore, fibronectin is important to establish cell-matrix adhesion and to guide cell migration. (Bruce Alberts 2002).

Laminins

Laminins are a family of cross-shaped glicoproteins with a central role in the formation, organization and physiology of basement membranes. Their C-terminal domain binds to cell surface receptors and is thereby responsible for cell anchorage and for the initiation of specific outside-in and inside-out signals. In contrast, their N-terminal domain interacts with proteins of the extracellular matrix to secure the basement membrane to the underlying mesenchymal tissue (Aumailley, El Khal et al. 2003).

Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains, composed of repeating disaccharide units, generally covalently linked to protein to form proteoglycan molecules. The repeating

units consist of a hexose or a hexuronic acid, linked to a hexosamine (six-carbon sugar containing nitrogen). (Varki A 2009). Polysaccharide chains are too stiff to fold up into the compact globular structures and are also strongly hydrophilic. Thus, GAGs tend to adopt highly extended conformations that occupy a large volume relative to their mass and form hydrated gels in the extracellular space (Bruce Alberts 2002). In other hand, proteoglycans are any protein with one or more covalently attached glycosaminoglycan chains. Proteoglycans are found on the surface of cells,where they function as co-receptors to helping cells to respond to environmental stimulation (Varki A 2009).

Considering the major events that take place at the matrix, is important to keep in mind that each of these components has a modular construction composed of domains that contain binding sites to one another and to receptors on the cell surface. Thus, the various components interact to form an interconnected network that is bound to the cell membrane (Karp 2009). An overview of the macromolecular organization of the ECM is represented in figure 1.1. In general, proteoglycans in ECM form a "ground substance" in which fibrous proteins are embedded. GAG molecules resist to compressive forces on the matrix and collagen fibers provide strength and help to organize the ECM while the resilience is given by elastin fibers. Finally, many glycoproteins and proteins, such as fibronectin and laminin, provide specific binding sites for cell adhesion (Bruce Alberts 2002).

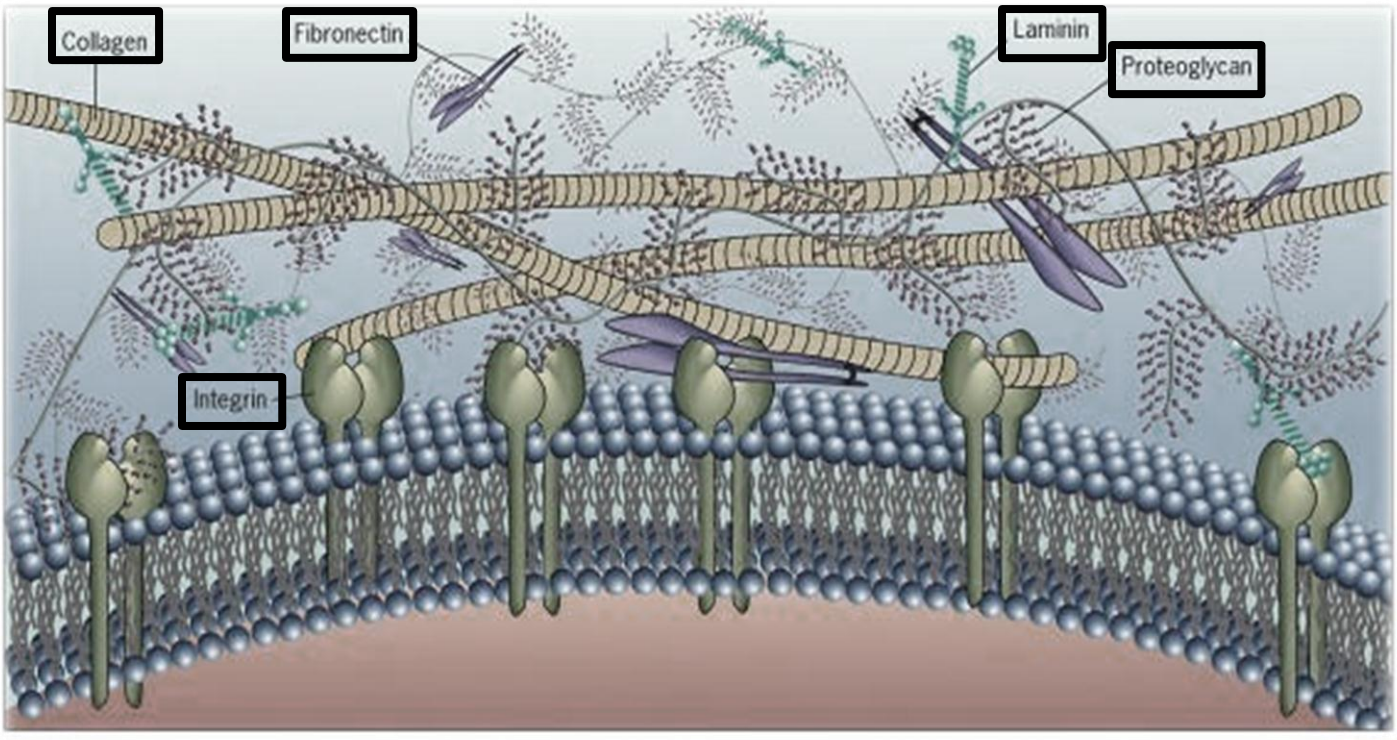


Figure 1.1- An overview of the macromolecular organization of the extracellular matrix (adapted from (Karp 2009))

1.2. ECM and their function

The high diversity and complexity of the ECM turns it a multi-functional network able to influence multiple biochemical and mechanical cellular processes. ECM provides an adhesive substrate for migratory cells, defines tissue architecture but also tissue boundaries and limits, constituting a physical barrier towards invasive cancer cells (Bronzino 2006, Eggeblad and Werb, 2002). Additionally, the ECM serves as efficient co-receptor and as a reservoir for sequestration and delivery of many growth factors. The ECM components activates, through interaction with cell surface receptors, intracellular signaling, modulating cell adhesion, migration, differentiation, growth, proliferation and apoptosis. Some of these ECM functions are illustrated in figure 1.2, although, a detailed view will be followed in section 1.3.

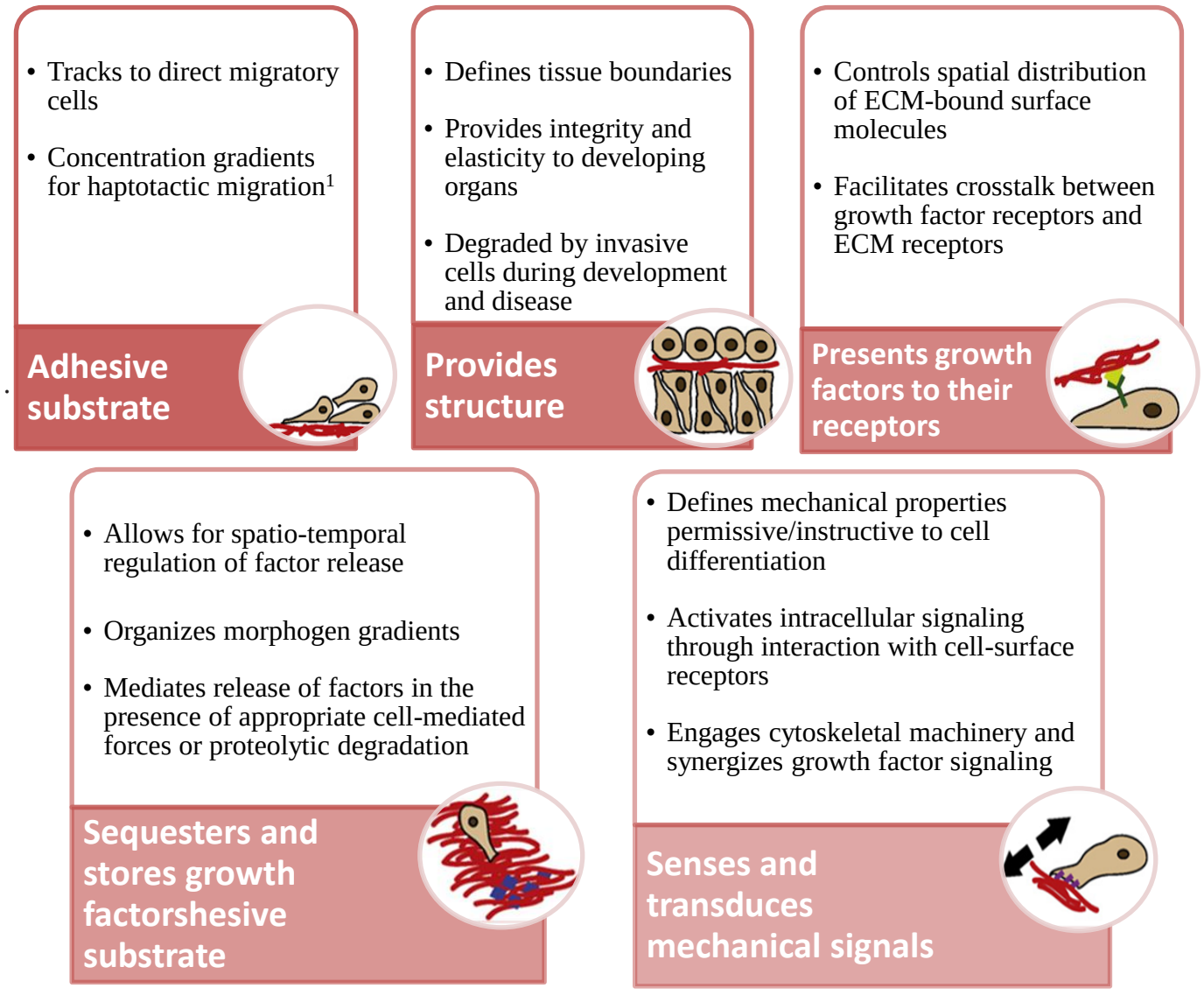


Figure 1.2- Functions of the extracellular matrix (adapted from (Rozario and DeSimone 2010))

1.3. ECM-Cells - A dynamic system

Either continuously or at certain phases or their lives, every cell makes close contact with the surrounding ECM components. These interactions are established and maintained through cell surface receptors that recognize and bind to specific aminoacid sequences within ECM. Integrins are the major mammalian cell receptors engaged to adhesion to extracellular matrix components and once activated they play key roles in immune response, development, homeostasis, leukocyte trafficking and cell migration, amongst many others. Integrins are αβ heterodimers comprising 8 β and 18 α subunits, so far known to assemble into 24 distinct dimers able to bind to many ligands (Hynes 2002). Figure 1.3 illustrates the major ECM ligands for β_1 integrin, and summarizes some of the co- receptors for each of these ECM molecules (Lal, Verma et al. 2009). While fibronectin has the tripeptide RGD that promotes cell adhesion, other amino acid adhesive motifs have been found in ECM molecules such as laminin, entactin, thrombin, tenascin, fibrinogen, vitronectin, collagen type I, collagen type VI and osteopontin (Anthony Atala 2010).

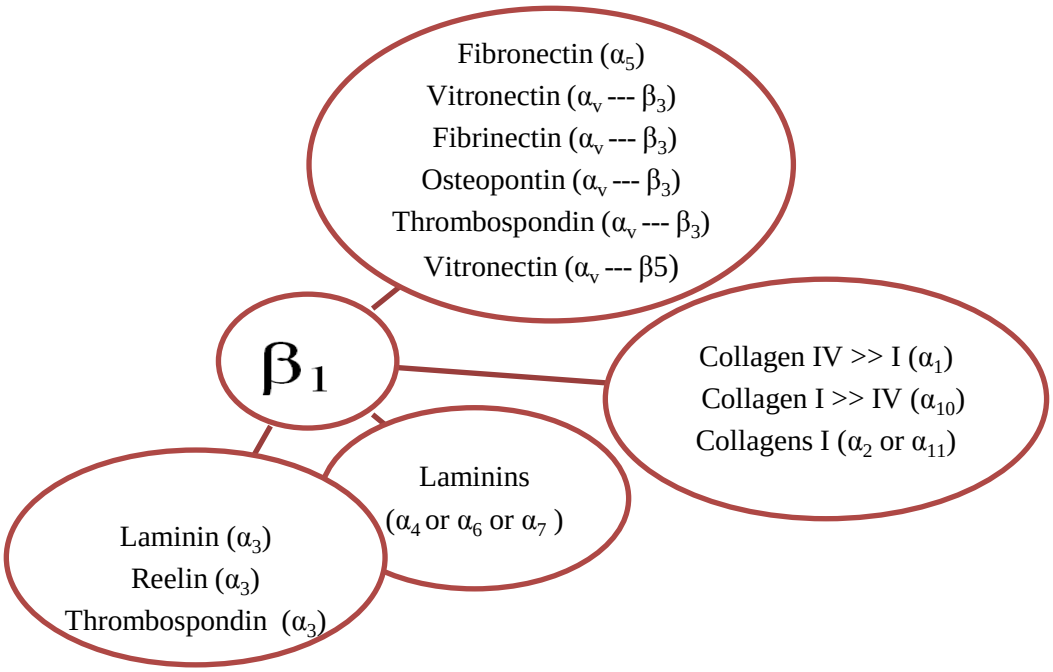


Figure 1.3 - β_1 integrins ligands and co-receptors (adapted from (Lal, Verma et al. 2009)).

Other cell-surface receptors have been identified such as, proteoglycan receptors, the elastin-laminin receptor, the epidermal growth factor receptor (EGFR) and the discoidin domain receptor family number 1(DDR1). Likewise integrins, they can also participate in cell adhesion and migration, although the signaling downstream receptor-ligand binding is less well known (Varki A 2009).

The interactions between ECM molecules and their receptors can transmit, directly or indirectly, signals to molecules within the cell, leading to a cascade of events that may culminate with the expression of a variety of genes involved in cell adhesion, migration, proliferation, survival, differentiation and death (Anthony Atala 2010). Some of the major signaling events important in these cellular processes are discussed below, and illustrated with some examples.

1.3.1. Adhesion and Migration

Cell migration is an essential process which requires precise regulation and integration of multiple signaling pathways. It is fundamental in homeostatic processes such as immune response and regeneration but also for cancer cell invasion and metastasis. Hence, ECM serves as the molecular scaffold for cell adhesion and migration, two processes that are closely related.

Generally, polarized and adherent cells start to migrate upon formation of a protrusion, located at the front of the cell, and which defines the direction of movement. After that, adhesion receptors located at the basal surface of the cell bind to ECM ligands and these sites of cell-ECM attachment serve as traction points for migration but also stabilize the protrusion via structural connections to actin filaments. Finally, the cells contract from the rear edges towards the nucleus and the adhesion receptors are released, detaching the cell from the previous rear attachment sites (Janik, Litynska et al. 2010).

As mentioned before, many of the known cell-matrix adhesion receptors are integrins, a major family of cell–cell and cell–ECM adhesion proteins, which can directly induce biochemical signals inside cells. These interactions will lead to cytoskeleton changes and will modulate cell morphology, cell migration and/or differentiation. For example, cell migration is promoted when fibronectin binds simultaneously to integrins through its cell-binding domain and to proteoglycan receptors through its heparin-binding domain (Dedhar 1999). In other hand, (Hohenester, Maurer et al. 1997), experiments have demonstrated that SPARC, a matricellular glycoprotein, is poised to act between the cell surface and the ECM, as residues identified for cell binding, inhibition of adhesion, of focal adhesions cluster on

one face of the protein, whereas collagen-binding regions lie opposite. Thus, SPARC can cause widespread de-adhesive effects, acting as an antagonist of integrins-ECM interactions. By analogy, tenascin-C, another matricellular glycoprotein, disrupts the adhesion of cells to fibronectin and prevents its binding to the signaling cell surface receptor, syndecan 4 (Scholze, GÖTz et al. 1996).

As shown before cell adhesion receptors play important roles in promoting migration. The establishment of strong adhesion inhibits migration, which is fastest at optimum adhesion strength: strong enough to support traction but weak enough to allow rapid detachment of the rear of the cell (Janik, Litynska et al. 2010).

1.3.2. Cell growth, Proliferation and Apoptosis

Cell cycle can vary from organism to organism, and represents the series of events leading to cell division. This mechanism has a central role in regulating the number of cells of a given tissue and thus, when the system malfunctions, excessive or defective cell divisions can result in severe diseases. Cell number depends on the balance between cell division and cell death.

Therefore, there are three fundamental processes that have to be referred: cell growth, cell division and cell death. Each mechanism is independently regulated by intracellular programs but also by ECM signal molecules. Accordingly, extracellular components can be grouped in three major classes (Bruce Alberts 2002):

- Mitogens, which stimulate **cell division**;
- Growth factors, which stimulate **cell growth** by promoting the synthesis of proteins/macromolecules and by inhibiting their degradation;
- Survival factors, which promote **cell survival** by suppressing apoptosis.

As previously mentioned, the growth factors that are arrested between the ECM components stimulate cell growth by accumulation of proteins/macromolecules, by increasing their rate of synthesis or by decreasing their degradation. One of the most important intracellular signaling pathways activated by growth factor receptors involves a kinase, called TOR. TOR is activated in response to increasing levels of nutrients and is required for the stimulation of growth but also survival under high-nutrient conditions (Morgan 2007).

Integrin-ECM binding can promote cell proliferation through multiple signaling pathways, many of which involve the activation of MAP kinase pathways, cooperating with growth factor signals (Guo and Giancotti 2004; Roth, Tweedie et al. 2010).

In other hand, the extracellular matrix can also arrest survival factors, required for a cell to survive. If deprived of such factors, the cell activates their intracellular program and dies by apoptosis. In contrast, binding activates signaling pathways that keep the death program suppressed, often by regulating members of the Bcl-2 family of proteins. For example, survival factors can stimulate the increased production of apoptosis-suppressing members of this family or can act by inhibiting the function of apoptosis-promoting members of the family (Bruce Alberts 2002).

Until now just positive regulators of cell-cycle, growth and survival have been discussed. However, tissues are also influenced by inhibitory extracellular signal proteins that inhibit organ growth. The best understood of these proteins is TGF-β that binds to cell-surface receptors and initiates an intracellular signaling pathway that leads to changes in the activities of gene regulatory proteins, such as Smads. These regulatory proteins bind to a specific DNA sequence and changes the transcription of genes encoding regulators of cell division and cell death (Schuster and Krieglstein 2002).

Apoptosis has been studied by many authors. For example, the neutrophil apoptosis stimulated by TNF-α is dependent upon β2 integrin-mediated signaling events involving the activation of the Pyk2 and Syk tyrosine kynases (Avdi, Nick et al. 2001). In other cell types, alterations in the ligand presentation by ECM can also regulate apoptosis. Curiously, studies have suggested that integrins ligation by soluble ligands can function as integrins antagonists and promote apoptosis rather than survival or proliferation (Vogel, Thomas et al. 2001).

1.3.3. Differentiation

Cell differentiation consists on developing into a specialized type of cell in response to specific environmental signals. Interaction of cells with ECM components, hormones, and growth factors is necessary to activate genes that are required for differentiation (Anthony Atala 2010). ECM may provide permissive and even instructive differentiation signals. Laminin alone has shown to promote specific fates in different tissues. Mouse and human stem cell precursors differentiate into neurons, astrocytes and specific glia on laminin but not fibronectin (Flanagan, Rebaza et al. 2006). Similarly,

multipotent embryonic lung cells can be induced to differentiate into smooth muscle cells on laminin (Nguyen and Senior 2006).

It has been suggested that morphogens and cytokines are displayed by ECM components and cells receive these signals as they come into contact with assembled ECM or when the associated factors are released by proteolysis (Rozario and DeSimone 2010). Thus, cell fate specification might result of the cross-talk between growth factor signaling and ECM. More examples can be given by tenascin-C which promoted glial cell differentiation via regulation of Wnt signaling. Additionally, Syndecan-4 has also been shown to influence neural induction in Xenopus via FGF and ERK signaling pathways (Kuriyama and Mayor 2009).

1.4. ECM in tissue engineering and regenerative medicine

Tissue engineering and regenerative medicine are an emerging multidisciplinary field that applies the principles of engineering and biology sciences in order to improve the health and quality of people wordlwide by the development of biological structures that restore, maintain or enhance tissue and organ function. There are three main approaches focus on: (a) the use of cells or cell substitutes that will replace cells that lost their function; (b) the delivery of tissue induced substances to targeted locations; and (c) growing cells in 3D scaffolds (Shin, Jo et al. 2003). Concerning to extracellular matrix, the third option has become increasingly important since the 3D organization and distribution of ECM components will play a pivotal role on support and guiding cells to grow.

Back through the past, Leonardo da Vinci was one of the first to comprehend that unsdertanding nature is a pre-requisite to engineer solutions (Moroni, Lorenzo et al. 2009). Centuries later, a lot has been done regarding mimicking or studying ECM for the development of scaffolds with the most appropriated characteristics. Focusing in one of the most serious cases of our days, organ transplantation is limited by the number of available donors, leaving thousands of people each year on a transplant waiting list. Therefore, tissue engineering and regenerative medicine have been evolved due to the necessity of restore or replace tissues and organs through the use of cells and scaffolds (Shin, Jo et al. 2003). Four major groups of scaffolds can be considered: (1) synthetic biodegradable polymers, (2)

naturally derived polymers, (3) hybrid scaffolds from synthetic and natural polymers, (4) acellular matrices derived from decellularized tissues.

The most commonly used scaffolds are constructed from the first two groups mentioned before (Chen, Ushida et al. 2000). In general, the cells isolated from a patient are cultured in a biocompatible 3D structure, which mimics the functional and structural characteristics of the native tissue. Although the potential advantages of these biomaterials, synthetic polymers present a lack of cell-recognition signals, leading to poor cell adhesion and affinity. Additionally, for a significant range of these polymers, the surfaces of the scaffolds are hydrophobic, restraining cell seeding, which leads to inappropriate cellular activity (Kim and Mooney 1998). In other hand, naturally-derived polymers are mechanically weak and unstable to maintain desired shapes (Chen, Ushida et al. 2000). Thus, hybrid scaffolds seem to compensate some of the limitations of the synthetic and of the naturally-derived polymers, but is being difficult to adjust the optimal combination of both.

Concerning to acellular matrices they also have their limitations, such as the variability amongst preparations and the inability to completely characterize the bioactive components of the material. Furthermore, acellular matrices are in general allogeneic – obtained from different individuals of the same species – or xenogeneic – obtained from an organism of different species – which can add potential risks of pathogen transmission (Lu, Hoshiba et al. 2011). Xenogeneic and allogeneic cellular antigens induce an inflammatory response that may lead to an immune-mediated rejection of the tissue due to recognition as foreign by the host. However, ECM components are generally conserved among species and are well tolerated even by xenogeneic recipients (Gilbert, Sellaro et al. 2006). Thus, the decellularization of the tissue will avoid the complex mixture of structural and functional proteins that frequently work as antigens, remaining the ECM and reducing the host immune response. Some examples of the use of acellular matrix as biological scaffolds can be given.

For example, is well known that several persons worldwide are waiting a donor heart. A bioartificial heart is an option that needs to respond to: engineering of a construct to provide adequate architecture; appropriate cell composition; and maturation of the construct to develop pump function. Shin and coauthors) have been focuses on the decellularization of hearts, preserving the underlying extracellular matrix, and posterior reseeding with cardiac or endothelial cells (Shin Jo et al, 2003). With sufficient maturation, and after physiological load and electrical stimulation, pump function was detected (equivalent to about 2% of adult or 25% of 16-week fetal heart function). In fact, the study was limited to rat hearts but the authors have successfully applied decellularization to porcine hearts, showing the

potentiality of the construct when scaled to hearts of human size and complexity. The same technique was also applied to other mammalian organs such as lung, liver, kidney and muscle. In other approach, the same authors hypothesized that acellular liver-derived biomatrix (LBM) may provide appropriate cues to sustain liver-specific functions of hepatocytes. After analyzing porcine-derived LBM, the results showed that LBM may be useful for hepatic tissue engineering application due to their ability to directly support hepatocyte adhesion, viability, and long-term liver-specific functions (Shin, Jo et al. 2003).

Many other researches proved that different tissues or organs could be successfully decellularized and their application is increasingly frequent in regenerative medicine and tissue engineering strategies (Shin, Jo et al. 2003). The most common clinical products composed of decellularized tissues are harvested from a variety of allogeneic or xenogeneic tissue sources, including dermis, urinary bladder, small intestine, pericardium and heart valve as shown in table I.

As showed before, scaffolds improvement and development is definitely an important area of Tissue engineering. However, deconstructing mature ECM and understanding its complex functions can be also very interesting. For example, there are several diseases, as multiple sclerosis or spina bifida, which can lead to a variety of complications in urinary bladder, such as small capacity, high pressure and poorly compliant. For this reason, surgical intervention is a therapeutic alternative to restore renal function, continence, resistance to infection, among others. Sleeman and Steeg evaluated the ability of bladder acellular matrix to support the individual and combined growth of primary porcine bladder smooth muscle and urothelial cells (Sleeman and Steeg, 2010) . The influence of urothelial cells on smooth muscle cell behavior on bladder acellular matrix indicates that this matrix may contain some inductive factors that sustainbladder cell–cell and cell–matrix interactions.

In conclusion, acellular-ECM studies may have two main goals: a) provide a good solution for scaffolds of several types of tissues or organs; b) provide a closer approximation to the *in vivo* environment and thereby facilitate the identification of some of the components and phenomena of the tissue or organ. Another future interesting approach can be the modification of acellular matrices with bioactive peptides and proteins that will extend well binding motifs to promote cell adhesion (Sleeman and Steeg 2010).

Table I - Examples of clinical products composed of decellularized tissues. (from (Shin, Jo et al. 2003))

Product (Manufacturer)	Tissue Source	Application Focus
AlloDerm® (Lifecell Corp.)	Human dermis	Soft tissue
AlloPatch HD™, FlexHD® (Musculoskeletal Transplant Foundation)	Human dermis	Tendon, breast
NeoForm™ (Mentor Worldwide LLC)	Human dermis	Breast
GraftJacket® (Wright Medical Technology Inc.)	Human dermis	Soft tissue, chronic wounds
Strattice™ (Lifecell Corp.)	Porcine dermis	Soft tissue
Zimmer Collagen Repair Patch™ (Zimmer Inc.)	Porcine dermis	Soft tissue
TissueMend® (Stryker Corp.)	Bovine dermis	Soft tissue
MatriStem®, Acell Vet (Acell Inc.)	Porcine urinary bladder	Soft tissue
Oasis®, Surgisis® (Cook Biotech Inc.)	Porcine small intestine	Soft tissue
Restore™ (DePuy Orthopaedics)	Porcine small intestine	Soft tissue
FortaFlex® (Organogenesis Inc.)	Porcine small intestine	Soft tissue
CorMatrix ECM™ (CorMatrix® Cardiovascular Inc.)	Porcine small intestine	Pericardium, cardiac tissue
Meso BioMatrix™ (Kensey Nash Corp.)	Porcine mesothelium	Soft tissue
IOPatch™ (IOP Inc.)	Human pericardium	Opthalmology
OrthAdapt®, Unite® (Synovis Orthopedic and Woundcare Inc.)	Equine pericardium	Soft tissue, chronic wounds
CopiOs® (Zimmer Inc.)	Bovine pericardium	Dentistry
Lyoplant® (B. Braun Melsungen AG)	Bovine pericardium	Dura mater
Perimount® (Edwards Lifesciences LLC)	Bovine pericardium	Valve replacement
Hancock® II, Mosaic®, Freestyle® (Medtronic Inc.)	Porcine heart valve	Valve replacement
Prima™ Plus (Edwards Lifesciences LLC)	Porcine heart valve	Valve replacement
Epic™, SJM Biocor® (St. Jude Medical Inc.)	Porcine heart valve	Valve replacement

1.5. ECM in cancer and metastasis

In cancer, cells may proliferate uncontrollably and give rise to neoplastic growth or tumours. Epithelial tumours can be distinguished into two types depending upon the ability of their cells to invade the adjacent extracellular matrix. If the tumour cells remain restricted to the primary site of the tumour, it is classified as benign, if tumour cells had however cross their limits and invaded the underlying stroma, the tumour is classified as malignant or cancer. However, is also important to focus another event that may occur as a consequence of cancer: the metastases formation. It is estimated that more than 90% of deaths from cancer are due to the direct or indirect effects of metastases (Sleeman and Steeg 2010). Metastasis is the spread of these abnormal cells from one place of the body to another non-adjacent local, by the ability to penetrate (intravasate and extravasate) the walls of lymphatic and/or blood vessels. Therefore, they invade the new organs, forming a metastatic cancer with cellular and molecular characteristics that are not necessarily like the primary tumour.

After several studies, a number of structural and functional differences have been detected by studying cells transformed by oncogenic viruses (Rastogi 2003):

- Genetic change: cancer cells have revealed that they lack intercellular adhesion and their cell surface becomes convoluted giving rise to villose structures; the nucleous is enlarged and irregular; the chromosomes swell up and the number of chromosome sets increase owing to the growth if transformed cells; the nucleoplasm undergoes drastic changes; the DNA is largely in uncoiled state, among other genetic changes.
- Cytoplasmatic inclusion: cancer cells have a reduced Golgi apparatus and the cytoskeletal elements like microtubules also tend to be disorganized. Thus, with this and other examples, the overall morphology of cancer undergoes alteration.
- Uncontrolled proliferation: cancer cells do not respond to various signals that regulate cell and consequently they proliferate at an uncontrollable rate. Tumor environment emerges as one of the major factors that determine the behavior of malignant cells since the interaction between cancer cells and ECM, among other factors, create a condition that promotes tumor growth and protect it from immune attack. Remodeling of ECM through altered expression of molecules is essential for local tumor cell invasion and the formation of metastases.

Therefore, ECM decellularization might be studied in order to analyze the components and structure of ECM, leading to the understanding of the most important phenomena of the disease. For example,

some proteoglycans, namely lecticans, have been identified as modulators of fibrosis and cancer growth, probably by up-regulation of p21, an inhibitor of cyclin-dependent kinases (Santra, Eichstetter et al. 2000). Similarly, it was shown, in breast cancer patients that another proteoglycan named lumican is secreted by fibroblasts adjacent to the tumor but not by cancer cells. Low levels of lumican expression were associated with a shorter time to progression of the disease and poorer patient's survival suggesting also a role of these proteoglycans in a host reaction to tumor invasion (Leygue, Snell et al. 2000). In contrast, versican, a large ECM proteoglycan that has strong anti-adhesive effects seems to facilitate local tumor invasion and formation of metastasis by decreasing ECM-cell adhesion (Sakko, Ricciardelli et al. 2001).

Angiogenesis has also an important role in the metastatic process. SPARC and other matricellular proteins were suggested to present regulatory effects on this event. An overall inhibitory function for SPARC in vascular growth is indicated by the enhanced fibrovascular invasion of sponges in SPARC-null mice (Bornstein and Sage 2002). The role of ECM in cooperation with tumor suppressors has also been studied. For example, evidences showed that ECM could modulate the cellular response to genotoxic stress as well as the function of the key tumour suppressor genes, such as p53 and Rb.

Besides ECM, the tumor microenvironment is constituted by fibroblasts, inflammatory cells, among others, which also play an essential role. For example, during healing, fibroblasts infiltrate the wound and remodel the microenvironment, orchestrating angiogenesis and cell proliferation to repair the tissue. However, whereas wound healing is a transient response, the tumor microenvironment remains in an activated state, which lead to the model that tumors behave like "wounds that never heal" (Bornstein and Sage 2002).

These are just few examples. Much more can be cited to demonstrate the role of ECM in cancer. However, the most important idea is that the study of ECM and its components is essential to understand very specific events that occur at the cellular level. Investigation of this process might provide new insights into the mechanism of tumourigenesis, leading to new therapeutic targets.

B. The Decellularization

1.6. The Decellularization Methods

The decellularization methods can vary as according to the tissues and organs of interest. The efficiency of cell removal from a given tissue is dependent on the origin of the tissue and the specific methods that are used. The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM. The distinct decellularization methods can be grouped as physical, chemical non-enzymatic and chemical enzymatic and being used in combination. Each of these methods will be herein briefly explained.

1.6.1. Physical Methods

Decellularization of tissues by the most popular physical methods includes **freezing-thawing,** and **mechanical or ultrasound (sonication) agitation**. Generally, physical methods are used as a first step for decellularization, followed by the addition of chemicals among other substances. Thus, the impact and effect of these methods as an exclusive technique for decellularization cannot be usually described.

In a study for a autologous ECM scaffold Hoshiba and collaborators (Lu, Hoshiba et al. 2011) used six cycles of freeze-thaw for decellularization. This treatment followed by NH_4OH aqueous solution and other detergents incubation, efficiently removed cell membrane and most of the DNA content. Since long time ago, other researchers have been also using snap freezing for decellularization of tendinous and ligaments (Jackson, Grood et al. 1988; Roberts, Drez et al. 1991; Bechtold, Eastlund et al. 1994) or of acellular nerve grafts (Gulati 1988). The rapid freezing forms intracellular ice crystals which disrupt cell membrane, allowing then tissue decellularization.

Mechanical or ultrasound (sonication) agitation can also cause cell lysis. This technique is often used simultaneously with chemical methods to maximize the removal of the remaining cell debris. In their experiments for decellularization of porcine aortic valves Joyce and collaborators expose the samples to various chemicals, under continuous agitation (Liao, Joyce et al. 2008). The major pitfall of this technique is that if not used the optimal speed agitation, the ECM components can be fractured.

1.6.2. Chemical: non-enzymatic methods

Chemical non-enzymatic methods that help in decellularization of tissues or organs are widely used. They can be divided in different groups according to their composition. For example, **alkaline and acid treatments** are used with the goal of solubilization of cytoplasmic components as well as to remove nucleic acids such as RNA and DNA. For five different ECM scaffolds, Freytes and collaborators compared the efficiency of decellularization before and after peracetic acid treatment (PAA) (Freytes, Badylak et al. 2004). In these experiments was shown that PAA can effectively disrupt cell membranes and also intracellular organelles. The effects of PAA were also studied by other researches, showing that this treatment did not affect the content of fibronectin and of laminin (Hodde, Record et al. 2002) and that even preserved ECM-arrested growth factors integrity and function (Hodde, Record et al. 2001)

Considering the experiments of Hodde and collaborators after treatment with uronic acid, the ECM retained many of the native GAGs such as hyaluronic acid, heparin, etc (Hodde, Badylak et al. 1996). To find a mean of bladder augmentation that would avoid the complications encountered with the use of bowel segments, Probst et al developed the bladder acellular matrix to be used as a homologous graft (Probst, Dahiya et al. 1997). For this purpose, the bladder was treated with sodium chloride containg

DNAse and then treated with sodium desoxycholate. The results revealed an acellular matrix with an intact structure of collagen and elastin.

Non-ionic or ionic detergents also represent chemical non-enzymatic methods. Triton X-100 is the most widely **non-ionic detergent** for decellularization, acting by disruption of lipid-lipid and lipid-protein, while protein-protein interactions remain intact. In a histological evaluation of decellularized porcine aortic valves (Grauss, Hazekamp et al. 2005), cellular material was still found in the adjacent myocardium and at the aortic valve. This treatment also led to a nearly completely loss of the GAGs content and evident decrease in the fibronectin and laminin composition. As final result, the treatment changed ECM constitution, which could lead to problems in valve functionality, cell growth and migration.

For other tissues, such as tendon (Cartmell and Dunn 2000), blood vessels (Dahl, Koh et al. 2003) and ligaments (Woods and Gratzer 2005) Triton X-100 was shown as non-completely effective removing method of cellular material. Studies of decellularization of bovine pericardial tissue used three different treatments in which two of them were surfactant tridecyl alcohol ethoxylate (ATE) and Triton X-100. On both protocols, more than 95% of the DNA content was removed compared to the native tissue but was also showed a reduction of GAGs content (Mendoza-Novelo, Avila et al. 2011).

On the other hand, sodium dodecyl sulfate (SDS) is the most commonly used **ionic detergent** (Chen, Ho et al. 2004; Hudson, Zawko et al. 2004; Lin, Chan et al. 2004; Ketchedjian, Jones et al. 2005), among others as sodium deoxycholate (Baiguera, Jungebluth et al. 2010) and Triton X-200. The ionic-detergents are frequently used in combination with other detergents, as zwitterionic detergent. SDS solubilizes cytoplasmic and nuclear cellular membranes and tends to denature proteins. Woods et al measured the effectiveness of three extraction techniques, in the development of a decellularized bone–anterior cruciate ligament–bone graft, using combinations of Triton X-100-SDS, Triton X-100-Triton X-100 and Triton X-100-TnBP (Woods and Gratzer 2005). In these experiments, Triton-SDS was the most effective at removing cell nuclear material and intracellular proteins such as vimentin. On the other hand, SDS tends to disrupt the native structure leading to a decrease of GAGs and loss of collagen integrity. Elder et al studied the effect of different techniques on the decellularization of tissue engineered articulate constructs (Elder, Eleswarapu et al. 2009). The results showed that SDS, at concentrations of 1% or 2%, is an effective treatment, leading to cell removal. An exposure to 2% SDS for 1 hour resulted in a 33% decrease in DNA content, while maintaining both GAG and collagen content. Further, an exposure to 2% SDS for 8 hour led to complete histological decellularization as well

as a 46% decrease in DNA content, although the reduction of total GAGs composition. Therefore, it is important to define an ideal balance between the time of exposure/concentration and the effect of these treatments on ECM integrity and cell removal.

Zwitterionic detergents, another group of chemical methods, exhibit properties of both non-ionic and ionic detergents and may include:3-[(cholamidopropyl) dimethyl ammo-nio]-1-propanesulfonate (CHAPS) and sulfobetaine-10 (SB-10) and -16 (SB-16). CHAPS treatments in decellularization of blood vessels reveled a effective cell removal accompanied by ECM disruption, likewise the results with non-ionic detergents, and with a collagen content similar to the native artery (Dahl, Koh et al. 2003). In decellularization of nerves, combined treatments of SB-10 and SB-16 with Triton X-200 had a less impact on ECM structure, when compared with a combination of these zwitterionic detergents with Triton X-100 (Hudson, Liu et al. 2004; Hudson, Zawko et al. 2004).

Cell lysis by osmotic shock can also be considered using **hypotonic and hypertonic solutions** such as deionized water or low ionic strength solution (Goissis, Suzigan et al. 2000; Woods and Gratzer 2005). Exposure to a hypotonic solution followed by a hypertonic solution induced cell lysis but not removal of whole cell remnants (Dahl, Koh et al. 2003). Therefore, this type of treatment requires in general the combination of enzymatic and other chemical treatments.

Ethylene-diamine-tetra-acetic acid (EDTA) and ethylene-glycol-tetra-acetic acid (EGTA) are examples of **chelating agents** that bind divalent metallic ions, such as Ca^{2+} and Fe^{3+}, disrupting cell adhesion to ECM. Experiments showed that divalent cations are essential for binding of collagen and fibronectin at Arg-Gly-Asp receptor (Gailit and Ruoslahti 1988; Moore, Madara et al. 1994), being disrupted in the presence of chelating agents. These techniques are typically used in combination with enzymatic methods (Gamba, Conconi et al. 2002; Elder, Eleswarapu et al. 2009).

1.6.3. Chemical: enzymatic methods

Trypsin is widely used in decellularization protocols. This proteolytic enzyme cleaves peptide chains mainly at the carboxyl group of arginine and lysine, except when is followed by proline (Huber and Bode 1978; Teebken, Bader et al. 2000; Gamba, Conconi et al. 2002). Other enzymatic techniques include the use of **endonucleases** and **exonucleases** (Baiguera, Jungebluth et al. 2010). While endonucleases cleave the phosphodiester bond within a polynucleotic chain, exonucleases cleave the

phosphodiester bond at the end of the polynucleotic chain (Lieber 1997). Since these polynucleotic chains can be ribonucleotide or deoxyribonucleotide, this technique will lead to degradation of RNA or DNA. Zhou and collaborators studied the impact of heart valve decellularization using Trypsin/EDTA and Trypsin–detergent–nuclease treatments (Zhou, Fritze et al. 2010). The histological examination revealed efficient cell removal and no significant differences in the ECM histoarchitecture. The effects of decellularization by enzymatic methods are difficult to measure by itself since these treatments are commonly used in a combination of solutions.

1.6.4. Others

During application of decellularization techniques and consequently cell disruption, many proteases can be released and damage the native ECM structure. For this reason, in a variety of decellularization protocols, protease inhibitors are commonly used in combination with conditions that limit protease activity such as pH, temperature or time of exposure to some detergents. To minimize the contamination of the remaining ECM material antibiotic solutions might be also included (Ketchedjian, Jones et al. 2005; Woods and Gratzer 2005).

Considering the previously methods for decellularization is possible to inferred that an effective removal of cells, while minimizing the adverse effects to the remaining ECM, is obtained by a combination of methods. The most robust and effective decellularization protocol involves the combination of physical, chemical and enzymatic methods. As a first step, physical treatments can disrupt cell membrane and release cell components but they are generally insufficient to remove cell contents from ECM. Therefore, this method is generally combined with chemical treatments. Additionally, enzymatic methods provide bonds breaking, leading to separation of cellular components from the ECM. As an example of combination of methods, Baiguera and collaborators used 25 consecutive cycles of a detergent-enzymatic method for the decellularization of trachea (Baiguera, Jungebluth et al. 2010). Every single cycle consisted of osmotic and detergent treatment (sodium deoxycholate) followed by nucleic acid digestion (DNase-I). The results showed that cells and nuclear material were removed and only few chondrocytes were still visible. It was also confirmed that approximately 99% of DNA was removed while collagen fibers on the tracheal external surface were still intact.

1.7. Techniques to apply decellularization agents

Although aiming the efficient removal of cells and of nuclear material, the decellularization also needs to minimize adverse effects on the composition, biological activity and mechanical integrity of the extracellular matrix components. Thus, besides the type of method used, is essential to select the right technique to apply the decellularization agent.

The optimal application of the decellularization agent is dependent on the thickness and density of the tissue, and the intended clinical application of the decellularized tissue. While a pressure gradient technique facilitates chemical exposure and removal of cellular material by creating pressure that causes cell lyses, the agitation technique is also able to lyse cells but an aggressive agitation can disrupt ECM. However, these techniques might be ineffective while removing cells from thicker tissues.

Immersion is another technique, commonly used, which creates decellularization by submersion within a detergent solution under induced agitation. An experiment performed by Badylak, using immersion, showed that smaller samples could be decellularized, whereas thicker specimens exhibited remaining cells (Baiguera, Jungebluth et al. 2010). To circumvent this limitation, other researchers took advantage of the native vascular network of each organ by perfusing the detergent through this network and distributing it throughout the entire organ (Baiguera, Jungebluth et al. 2010). Therefore, immersion decellularization has been used previously to generate biological scaffolds in several thin and well-irrigated tissues including pericardium and vessel wall. However, for some tissues or organs, these approaches are ineffective at removing cellular material due to their density, mass and/or 3D architecture. Thus, vascular perfusion technique might be required, allowing a continuous supply and homogeneous distribution of the decellularization agents throughout the entire tissue.

2. Aim

The extracellular matrix consists of a meshwork of fibrous, non-fibrous proteins and proteoglycans and serves as a reservoir of many signaling molecules such as enzymes, growth factors, and cytokines. For many years, the ECM was thought to serve only as a structural support for tissues. Nowadays, is well known that the interactions established between ECM molecules and their receptors can modulate several cellular activities such as adhesion, migration, proliferation, survival, differentiation and death. The majority of the studies performed to evaluate molecular interactions established between cells and the surrounding extracellular matrix, uses animal-derived components that might be biochemically and mechanically different from their human counterparts. Therefore, to mimic as closely as possible the human ecosystem, we conceived an innovative approach that aims to address questions related to tissue regeneration and cancer invasion on human decellularized matrices. These matrices might in the future be also used as scaffolds and even functionalized with specific peptides for tissue engineering applications.

The major objective of this Master Thesis is the design and construction of a decellularization apparatus, herein designated as "Decellularization Cell" (DecCell) that using a perfusion technique might efficiently decellularize human tissues obtained from surgical resections at Hospital de São João, Porto. The DecCell aims to provide an efficient removal of cells and nuclear material of a given tissue, while maintaining the biological activity, mechanical integrity and composition of the extracellular matrix. The system will be designed according to specific features such as quality, reliability, functionality and safety and versatility, to be applicable to different sizes and tissues.

The possibility to develop an innovative matrix based on human ECM components in opposite to the classically used animal matrices will provide a significant development in the field. The use of both

autologous cells and autologous scaffolds would eliminate negative host responses in transplantation and lead to optimal tissue regeneration. Additionally the understanding of the cell-extracellular matrix interactions might provide new insights into the mechanisms of tumorigenesis and regeneration, leading to the development of new therapeutic approaches.

For these reasons, we believe that the design and construction of this dynamic perfusion system suitable for human matrices decellularization will be extremely useful for tissue engineering and oncobiology fields.

3. Materials and Methods

3.1. Designing

The design process of the decellularization cell was performed respecting the following order:

1. Design of handmade sketches;
2. Using ArchiCAD 14 to create 2D drawings;
3. Using 3D Studio Max Design 2011 to create the model;
4. Using MentalRay in 3D Studio Max Design 2011, to produce the architectural renderings of these models.

1. Design of handmade sketches

As a first approach, handmade sketches were created in order to construct a versatile apparatus that would allow the decellularization of human tissues of distinct areas, using a perfusion technique to maximize cell removal and minimize alterations of ECM properties. After many conversations established with colleagues from Biology, Pathology and Engineering the ideas started to gain form and a first 2D drawing was achieved. Figure 3.1 shows the handmade sketches of the decellularization agent container and the valve.

2. Using ArchiCAD 14 to create 2D drawings

After selection of the innumerous hand sketches, ArchiCAD was the program chosen to design the final 2D draft. ArchiCAD is an architecture software that provides specialized solutions for drawing common aspects of aesthetics and engineering. This software is able to create both 2D drawings and parametric 3D geometry. It provides a wide range of applications such as:

- 2D CAD (Computer Aided Design) software, which allows detailed technical drawings;
- 3D Modeling software, creating forms;
- Architectural rendering producing photorealistic pictures or videos;
- Building Information Modeling (BIM) software – an interactive tool which creates an interface between all the designs.

Besides these features, ArchiCAD was used to produce 2D vectorial drawings, such as sections and plans for each component of the decellularization cell. However, the capability of ArchiCAD to produce 3D modeling and rendering is insufficient to recreate the models that were imagined. Thus, 3D Studio Max Design 2011 was used due to its high performance in modeling. Figure 3.1 shows the 2D draw of the decellularization agent container.

3. Using 3D Studio Max Design 2011 to create the 3D DecCell model

3D Studio Max Design 2011 software provides powerful, integrated 3D, modeling, animation, rendering and compositing tools that enable designers quickly ramp up for production. In addition to its modeling and animation tools, this program also features shedders, dynamic simulation, particle system, brightness, normal map creation and rendering, global illumination, among others.

From the 2D vectorial drawings, the 3D modeling was initiated using extruding procedures. The perfusion chamber and their components, the decellularization agent containers and the decellularization chamber, were the first to be modeled. In this stage, forms and dimensions of the chambers were improved over the 2D drawings. Figure 3.2 shows the 3D model of the decellularization agent container.

Afterwards other objects were designed, such as the peristaltic pump, relay card and vacuum bottle, which helped to recreate a more realistic view of the decellularization apparatus. Even if, afterwards, these components were purchased and not built, their design had an essential role on the arrangement of the objects and their connections.

4. Using MentalRay in 3D Studio Max Design 2011, to produce the renderings

Rendering is the process of generating an image from a model, by means of computer programming. MentalRay is a render engine in 3D Studio Max Design 2011 which enables the creation of more genuine images. This feature can be used to create geometric elements at runtime of the renderer, procedural textures, bump and displacement maps, atmosphere and volume effects, environments, camera lenses, and light sources.

In order to create more realistic images, the appropriated materials for each component were selected, regarding their main features and functions. Therefore, while rendering, it was possible to achieve a more realistic view of the object, and consequently, a more realistic view of the whole composition – the decellularization cell. In this rendering process, the light source was carefully chosen since shadows could disturb the perception of the model. Figure 3.3 shows the decellularization agent container.

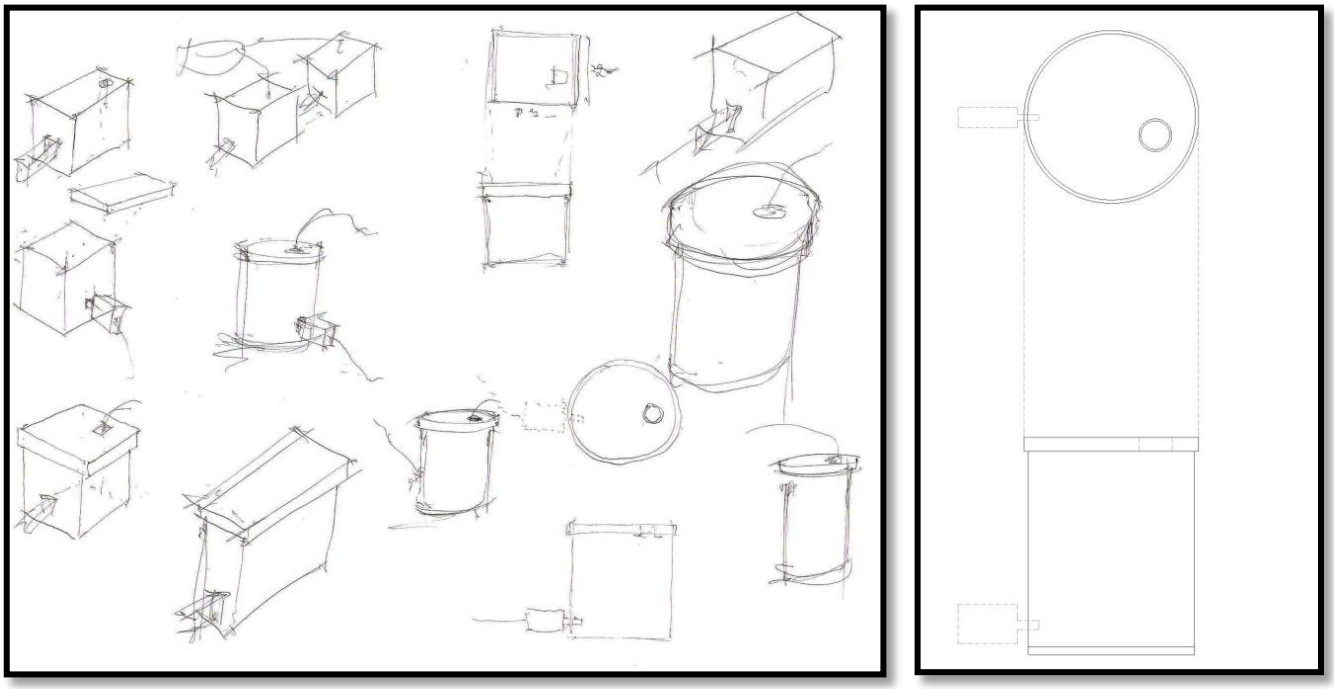


Figure 3.1 - – Design of the decellularization agent container coupled to valve. The handmade sketches (right) and the 2D drawings using ArchiCAD (left).

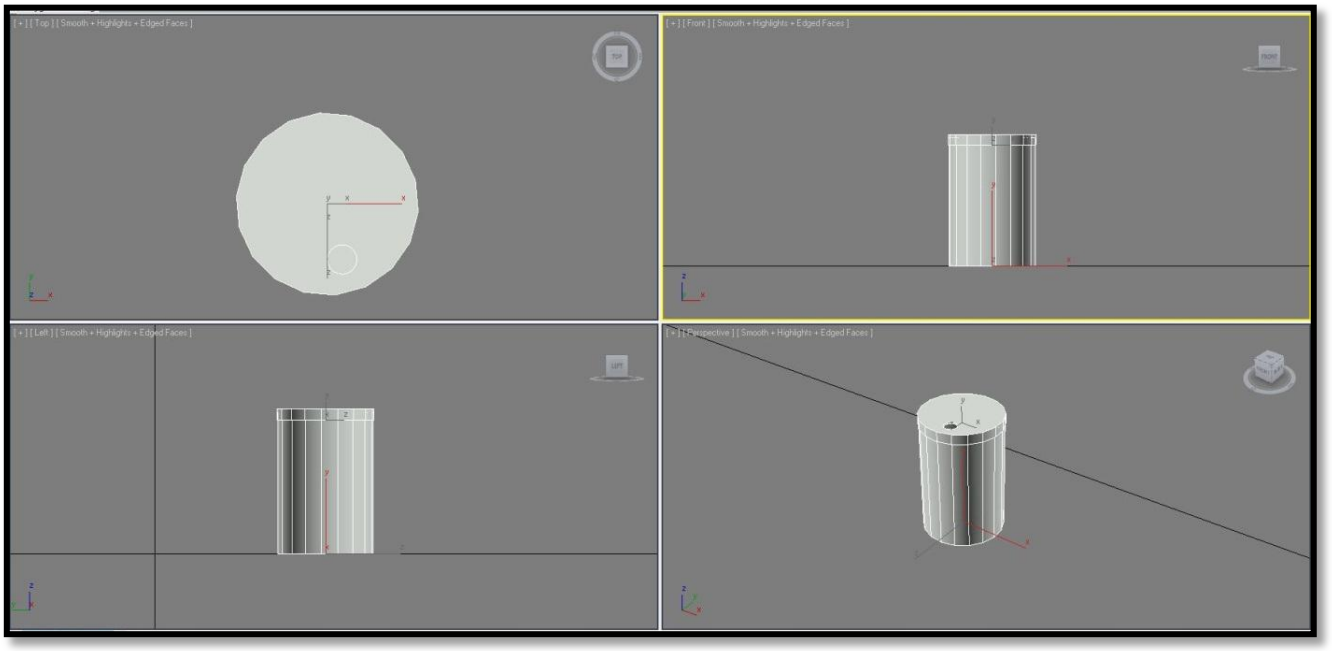


Figure 3.2 - – The 3D models of the decellularization agent container, with different views, using 3D Max Studio Design 2011

Figure 3.3 – The decellularization agent container and valve, after rendering

3.2. Constructing

Some components were purchased and their specifications can be followed in chapter 4-A.

- Computer, from Palmtop;
- Data Acquisition – DAQ, from National Instruments;
- Decellularization agents container, from Vidro Lab 2;
- Peristaltic Pump, from VWR International, Lda;
- Relay Card, from Paralab;
- Tubes, from VWR International, Lda;
- Vaccum pump, from VWR International, Lda;

On the other hand, the other components (further details in chapter 4-A) were designed and custom-made by experts in glass, acrylic and stainless steel.

3.3. Decellularization Cell Program

The decellularization cell was designed and constructed to be an automatic system in which the user would just need to interact with the software. Therefore, a program to control the valves and the flow rate of the decellularization liquid agents was created and developed in LabView®.

Labview® - Laboratory Virtual Instrumentation Engineering Workbench - is a platform and development environment for a visual programming language from National Instruments. This platform uses G programming, performed by wiring graphical icons on a diagram, which is then compiled directly to a machine code so that the computer processors can execute it. With LabView®, engineers and scientists can rapidly and cost-effectively interface with measurement, control hardware and analyze data. Figure 3.4 shows part of the program that was created (further details in chapter 4-B.)

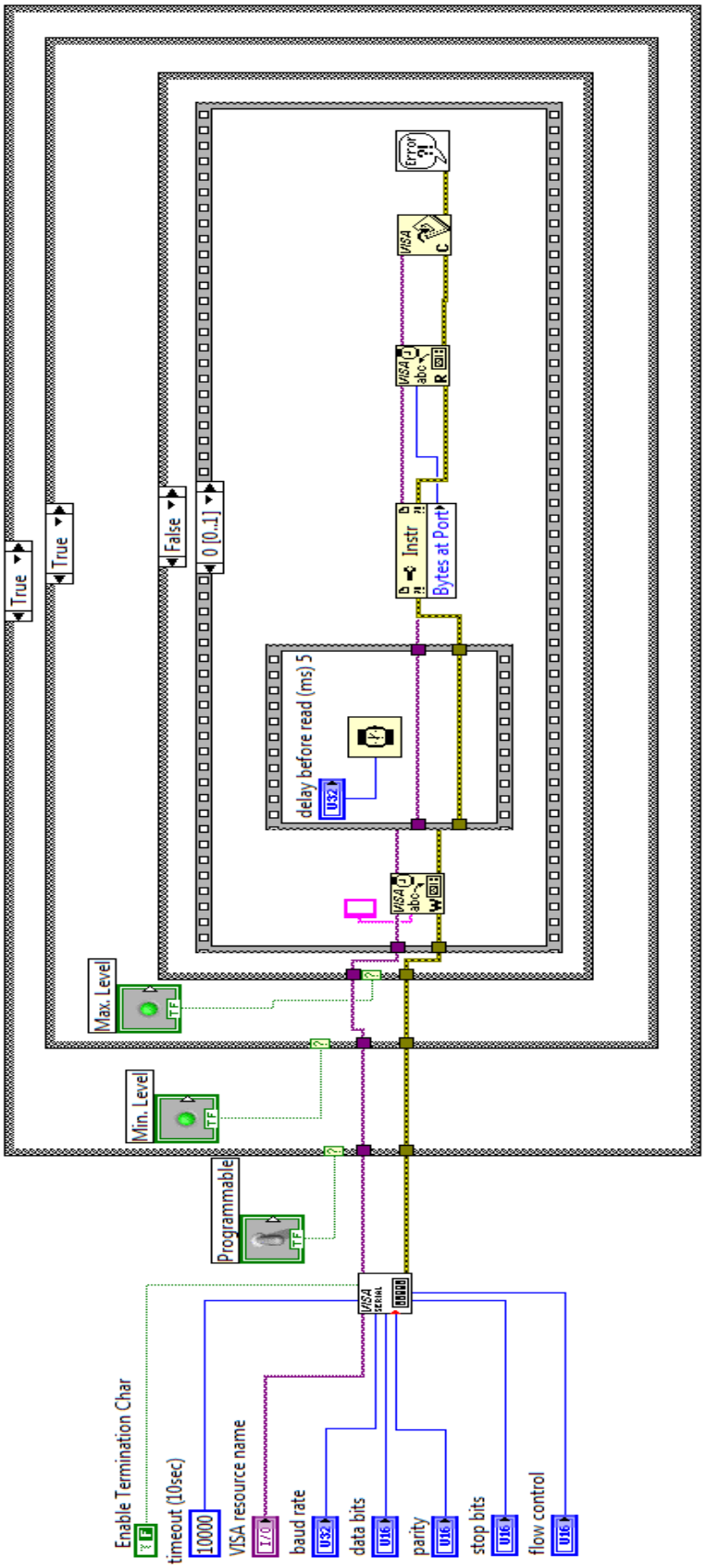


Figure 3.4 – Block diagram of the program created in LabView (further details in chapter 4-B)

4. Results and Discussion

A.

DecCell Design and Construction

Design informally refers to the establishment of a draft aiming the construction of an object or system. With this purpose, it was necessary to select the technique how the decellularization agent would be applied in order to assure efficient cell removal. After selecting the most appropriated decellularization technique, and after defining the required technical conditions, the distinct components of the decellularization cell were designed one by one.

All efforts were then made to turn the idea into reality. Some components were purchased and therefore, their specifications needed to be considered. Other components were designed and custom-made. The next section focuses on the reasons that led to the selection of the perfusion technique and illustrates the design and construction phases of each of the components that comprises the decellularization cell.

4.1. Technique selection

As previously mentioned, it is essential to select the right technique for the decellularization agent application. The most common techniques consist of:

- Pressure gradient;
- Agitation;
- Immersion;
- Vascular perfusion.

The optimal application of the decellularization agent has also to take into account tissue thickness and density. Since our decellularization cell was designed to provide maximal removal of cells, independently from the size and thicknesses of the tissues used, the pressure gradient and the agitation techniques were immediately excluded. Similarly, the most commonly used technique, based on tissue immersion was described to be ineffective in removing cellular material from ticker tissues. Thus, to circumvent this limitation, vascular perfusion was be used, allowing a continuous supply and homogeneous distribution of the decellularization liquid agents through the vascular network of some organs. However, the decellularization cell must be functional for a wide range of tissues and not only for vascular organs.

In synthesis, no technique fulfills the requirements of the decellularization cell: be functional for different sizes and thickness of tissues and be a dynamic system. Therefore, a new concept was created based on the techniques mentioned before – the perfusion technique.

Perfusion can be considered the act of a liquid poured through a specific tissue or organ. According to our design, the fluid will experience difference of pressures at both sides of the tissues; will not be immersed but will be continuously soaked and will be perfused. Therefore, it was possible to project a construct that could perform decellularization by perfusion technique.

4.2. DecCell components and their functions

The construction of a computer-assisted decellularization apparatus able, by using a perfusion technique, to decellularize distinct types of tissues, has been achieved according to the following drawing:

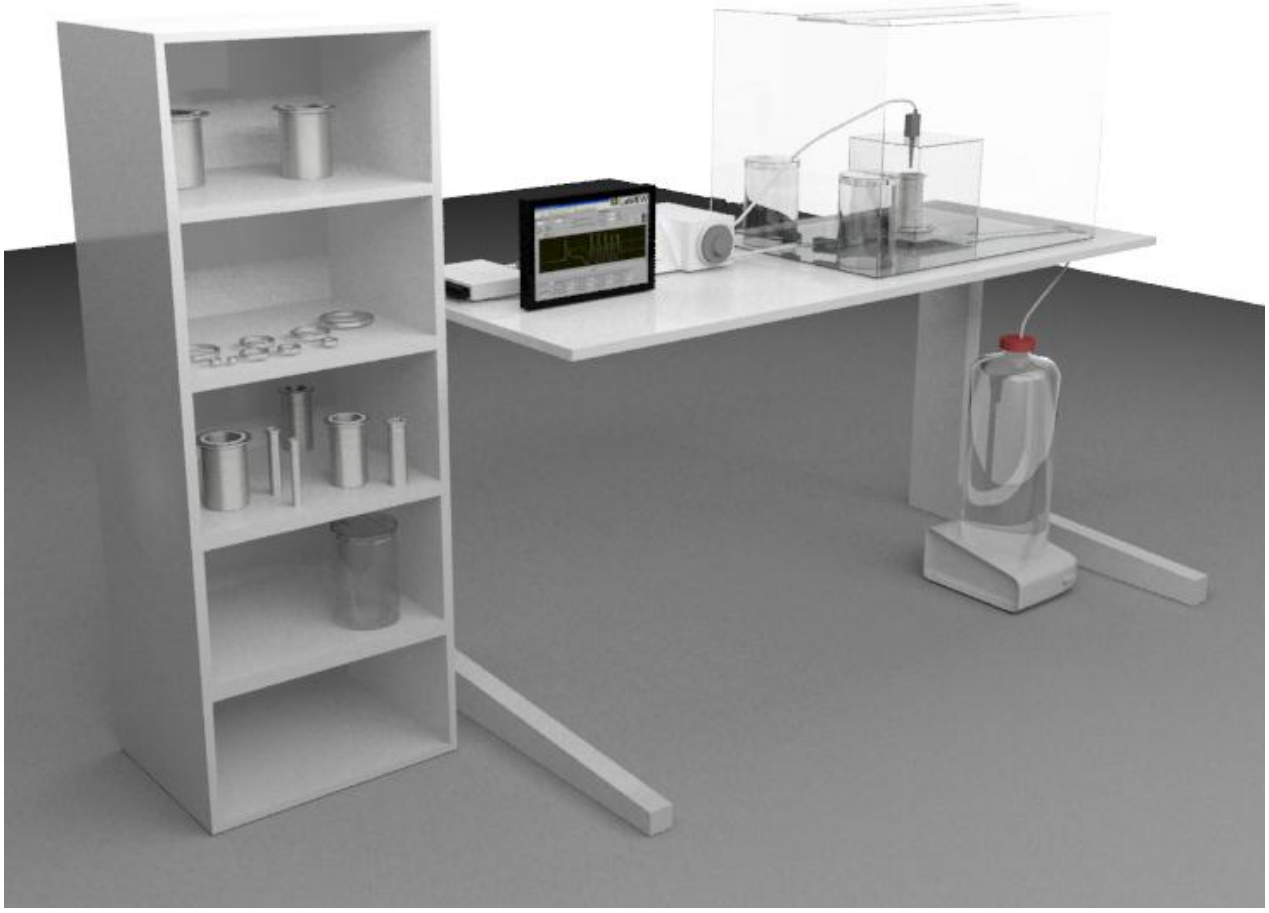


Figure 4.1 - The decellularization cell working area

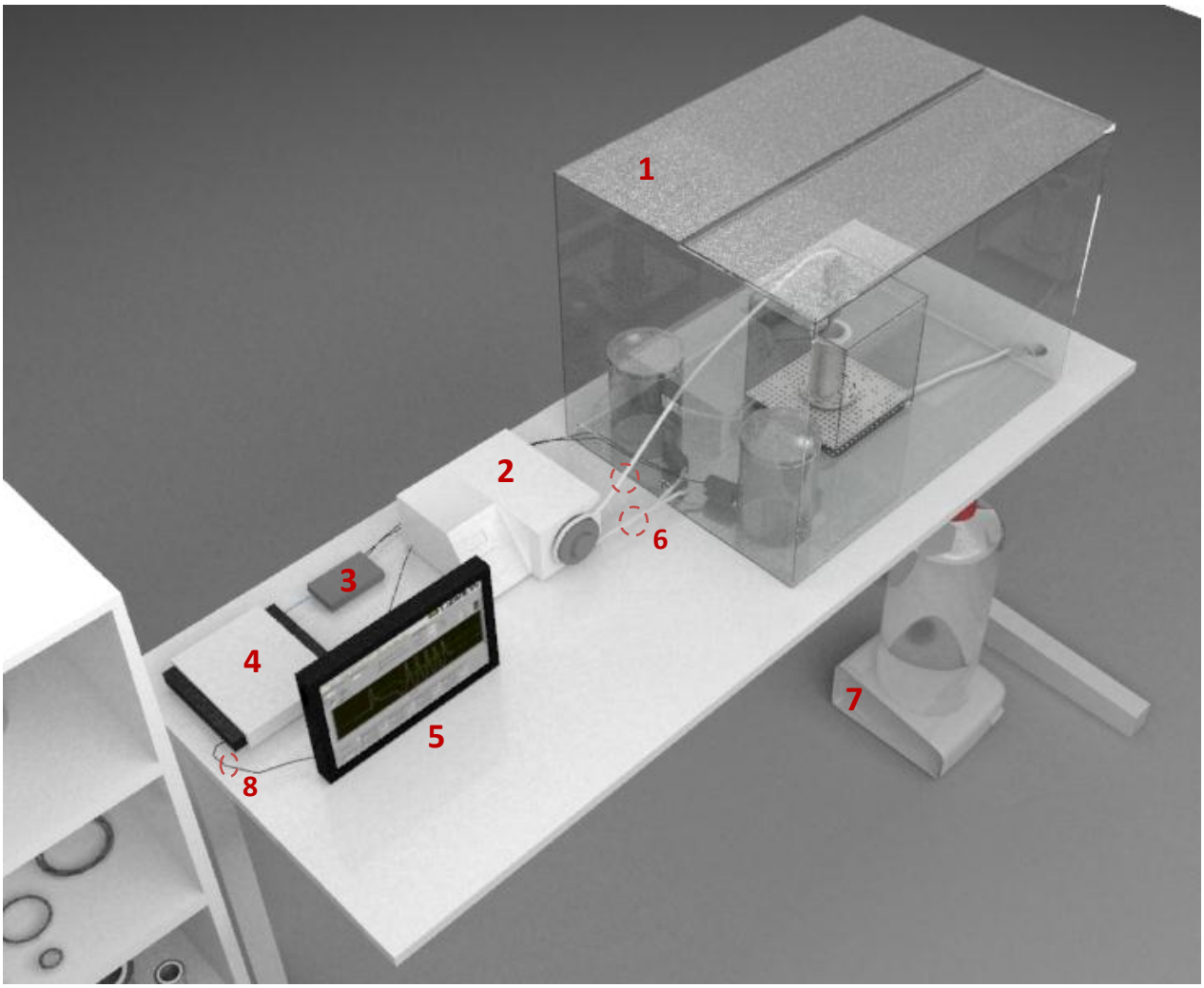


Figure 4.2 - The decellularization cell

Legend:

1 – Decellularization Chamber

2 – Peristaltic Pump

3 – Relay card

4 – Data Acquisition (DAQ)

5 – Computer

6 – Tubes

7 – Vaccum pump

8 – Connector cables

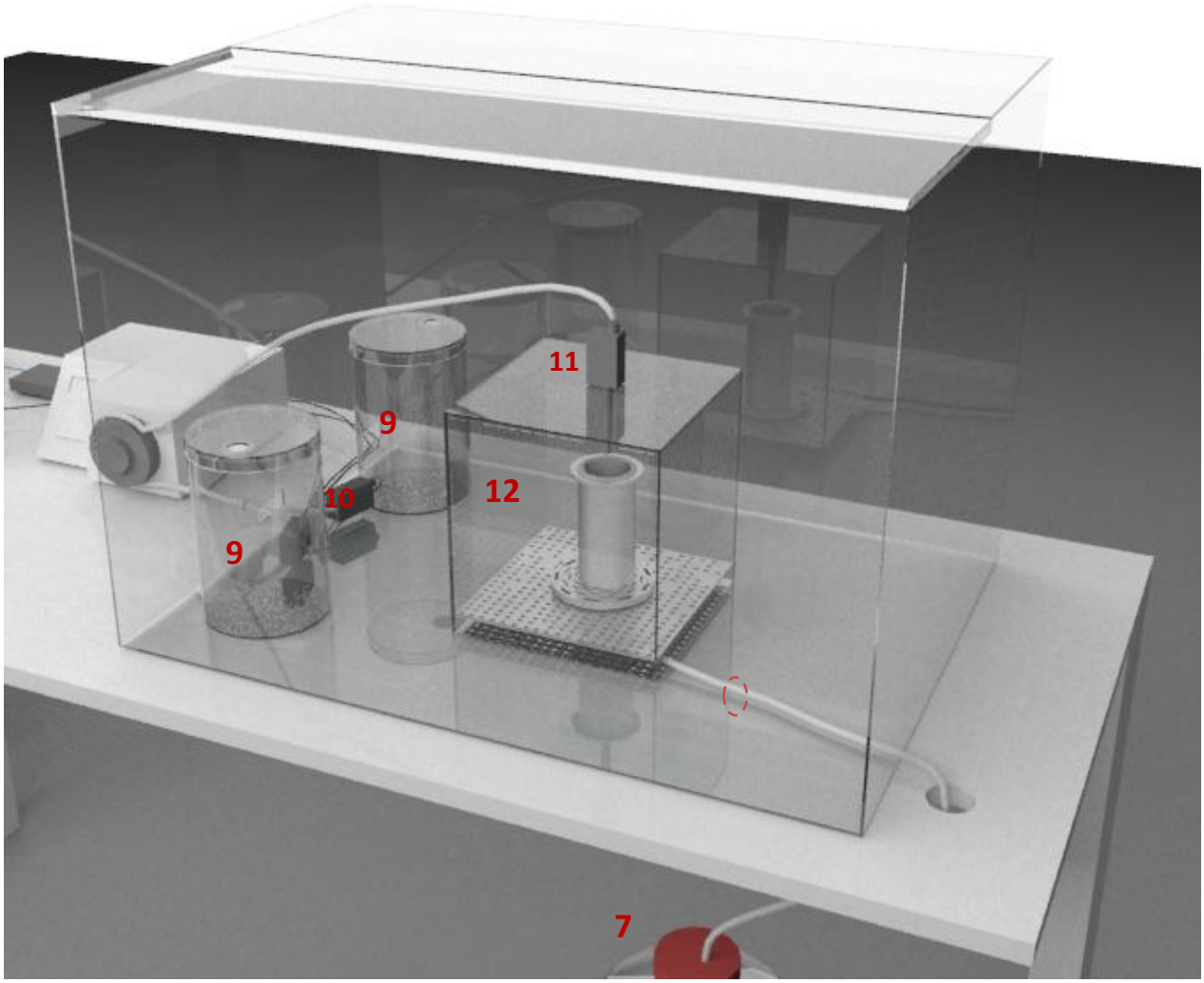


Figure 4.3 – The decellularization chamber

Legend:

7- Vacuum pump

9 – Decellularization agent container

10 – Valve

11 – Flow rate sensor

12 – Perfusion chamber

In this section, an introductory paragraph for each component, aiming its integration on the whole construct, is presented. For the majority of the components differences between the conception/design (*design*) and the final construction achievement (*construction*) are indicated. These differences correspond to improvements that have been introduced to the initial design scheme, in order to facilitate user manipulation, sample handling or maximize the decellularization efficiency. For other components that have not changed in comparison to the original design draft, no differences are annotated.

Decellularization chamber (1)

The decellularization chamber serves as a protection for the decellularization materials and tissues, providing an individual space for the process.

While designing

The chamber was designed in order to offer a local to displace the decellularization containers, the perfusion chamber and other materials that could be necessary along the process. It was conceived to possess lateral and back walls, floor, ceiling and a front door. When the authorized user would need to contact the inside part of the chamber, the front door was thought to be removed and placed horizontally in the space between the work table and the basis of the chamber. Additionally, if the worker just needs to readjust the position of the chamber components, the ceiling can be opened by pushing the anterior glass, reducing the ceiling to half of the size.

While constructing

The decellularization chamber was built in acrylic, due to its low cost in comparison to glass chambers. Following the design idea, the chamber was constructed with a floor, a ceiling and lateral back walls. These components are static. On the other hand, to facilitate handling, the front door was changed and thus, when the worker needs to contact the inside of the chamber the front door can be folded and placed in the upper part of the chamber.

Computer (5)

The computer has an important role since it will allow automation of the whole decellularization process, being able to process data, to control the flow of the decellularization agent crossing the sample

and to control the physical conditions of the decellularization cell. The DecCell software will thereby receive a specific task from the user, coordinating the response.

While designing

Using a specific designed program, it will be possible the automation and programmable controlling of the whole decellularization process. For example, by clicking in a computer button, the user can activate the system, initiating tissue decellularization. The software will send a signal, which ultimately leads to the opening of the valves. Additionally, the user can also regulate the flow rate of the process. Therefore, the computer will receive the signals from the sensor and will send a signal to the peristaltic pump that responds adjusting the flow rate of the decellularizatin agent that is perfusing the tissue. More details of this process can be followed in section 4.B.

While constructing

This computer had to fulfill some simple obligatory requirements:

- At least three USB interfaces: The 1st one to set the connection with the peristaltic pump, the 2nd to set the connection with the DAQ and the 3rd for a common approach, such as data pick up collection;
- An approximately 14'' display screen. Small enough to reduce the space occupied by the DecCell but with a sufficient size to be easily used;
- A computer processor and memory compatible with the requisites to implement LabVIEW® software;
- A trusted brand.

For these reasons, an Asus K43SV desktop computer was bought with the following specifications:

Processor: Intel Core i5-2410
Display screen: 14.0″ WSXGA (1366×768) LED
Graphics card: NVIDIA GeForce GT 520M / 540M with 1GB DDR3 VRAM
Memory RAM: 4GB DDR3
Hard Disc Memory: 500GB SATA
Interfaces: 3 x USB 2.0 ports

Operative System: Windows 7 Home Premium 64bits
Weight: 2, 44 kg
Autonomy: 4h00

Data Acquisition System (4)

DAQ is an acronym for Data Acquisition System and is a board which can convert signals (digital → analog or analog → digital) that are sent by the computer. For this reason, the DAQ is also connected in series to the relay card, allowing signal flow.

While constructing

The DAQ should be able to convert the signals received from the computer and to send them to the relay card. Since this equipment has a plug-and-play USB connectivity, this device is ideal for quick measurements and also versatile for more complex measurement applications. However the DAQ needs software to interface with the hardware (computer) and to collect, analyze, present and store the achieved measurements. Thus, this board has to be compatible with a variety of programming languages, including LabVIEW®.

For this reason, a National Instruments USB-6008 DAQ was bought, with the following specifications:

- 8 analog inputs (12-bit, 10 kS/s);
- 2 analog outputs (12-bit, 150 S/s); 12 digital I/O; 32-bit counter ;
- Bus-powered for high mobility; built-in signal connectivity
- Compatible with LabVIEW ;
- NI-DAQmx driver software and NI LabVIEW SignalExpress LE interactive data-logging software;

Further details of these elements can be followed in section 4.B.

Relay card (3)

The relay card is connected to the flow rate sensor as well as to the valves and to the DAQ. Therefore, the relay card directly interfaces with this distinct components and intervenes in the

opening of the valves and in flow rate control. The signal from the DAQ is received by the relay card data which supplies the valves or sensor, which in turn allows their functionality.

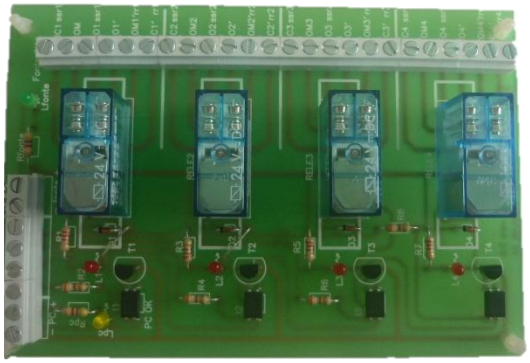


Figure 4.4 – Relay card from Paralab

While constructing

The relay card chosen has 24 volts due to its connection with the valves, which present a supply voltage of 24 Vdc.

Peristaltic pump (2)

The peristaltic pump is used in order to pump a variety of fluids, in this case the decellularization agents that will perfuse through the tissue sample, promoting and controlling their flow rate.

While designing

The fluid is contained within a flexible tube placed inside of a circular pump casing. This pump has a rotor that compresses the flexible tube and in its turn closes the part of the tube under compression, forcing the fluid to move through it. The fluid is totally contained in the tube, and never contacts directly with the pump, preventing contamination. Additionally, the gentle pumping action will supply accurate flow rates.

The peristaltic pump provides a keypad to regulate flow rate and also a small screen with high visibility. The keypad can be locked, preventing tampering or accidental changes. The peristaltic pump can also be connected to a computer that has a program to control the flow rate during the whole process, as considered for the decellularization cell.

While constructing

There are several peristaltic pumps providing different flow rates, however, they are usually manually controlled. Since the DecCell is desired as having an automatic control, the pump needs to present a programmable control. Additionally, because the ideal range of flow rate values will be just known after validation of the decellularization cell, and also because different tissues might require different flow rates, the pump also needs to respond to variable speed.

Therefore, Programmable Control (IP40) Peristaltic Pump, from VWR Fastload Peristaltic Pump collection, was bought, with the following features:

- User friendly keypad control;
- Two line alpha numeric display;
- Manual, analogue and digital control;
- Variable flow rate or speed;
- Six tube sizes;
- Single or repeat dosing;
- Calibrate for viscous liquids;
- Low maintenance brushless motor;
- Speeds from 10 to 250 rpm;
- Remote speed control;
- Remote stop / start / reverse;
- Volt free contacts or 3-36 V dc;
- RS232 computer signal interface.

Besides all these characteristics, among the programmable control (though the RS232 computer signal interface), this peristaltic pump also has manual control. Additionally, the pump is provided with six different tubes to be adaptable according to the desired flow rate which can vary from 0.9 ml/min to 1312 ml/min, as shown in table II.

Table II - Flow rate according with tube inner diameter for IP40 (from IP40 data sheet)

Tube inner diameter (mm)	0,8	1,6	3,2	4,8	6.3	8
Flow rate (ml/min)	0,9-19	2,8-70	11-270	24-587	38-937	53-1312

Tubes (6)

Tubes should allow the transport of the decellularization agents from the decellularization agent containers to the perfusion chamber where the tissue intended to be decellularized is located.

While designing

Since tubes will transport the decellularization liquid agents, it is important to have in consideration some important requirements. Compatibility is one of the most important characteristics that has to be considered. Tubes need to be elastomeric, in order to maintain the circular cross section after cycles of squeezing in the peristaltic pump and also to be chemically suitable. The tube life material is also important since its continuous degradation might release contamination elements into the perfusion agents.

Ordinary tubing is not designed for peristaltic pumping. The characteristics of the pump, including suction lift, pressure, life, flow rate and efficiency are all largely determined by the tubing. Thus, the tubes must be selected after pump acquisition to ensure compatibility. In general, the companies already have their own range of tubes for a specific pump. They also offer validation/testing packs, in which the user can analyze swelling, deterioration and chemical compatibility, among others.

The decellularization cell was designed considering a wide range of decellularization agents that could be more or less aggressive. For this reason, the selection of the tube can be done depending on the agent. However, it is already known that the tubes must minimize protein binding as well as other cellular material.

While constructing

The tubes must be carefully chosen after pump selection and in general, the companies already have their own range of tubes according to the specifications of the peristaltic pump. Regarding the requisites cited above, Tygoprene® Pump Tubing was selected, and their clear advantages must be considered:

- Flexibility, which allows maintenance of the circular cross section after cycles of squeezing in the peristaltic pump;
- Translucent in color, leading to a easiest location of the fluid within the tube;
- Excellent chemical resistance to a wide range of fluids, including bases or acids;
- Excellent resistance to ozone, oxygen and sunlight aging;

- Resistance to a wide range of temperatures;
- Suitable with a Pump life of over 500 hours.

According to the literature, the most commonly used tubes are PVC and silicone tubing. However, it is evident the longevity of Tygoprene® tubing when compared to those tubes as well as the excellent chemical resistance properties and permeability. This justifies our selection.

Decellularization agent containers (9)

The containers were designed in order to retain the decellularization liquid agents with minimal contamination.

While designing

As shown in figure 4.4 the containers have an air filter in their lid due to the necessity to contact with the atmosphere. If the filter would not be considered, the container could create vacuum pressure, leading to an inefficient process.

Each container is connected to an on/off valve, which controls the exit of the fluid. If the valve is open, the fluid will pass through the tubes, helped by the peristaltic pump and consequently leave the recipient. The containers are made by glass due to its biocompatibility for the wide range of decellularization agents. Additionally, a rubber was placed in the inner side of the lid, preventing any changes between the interior and exterior environment.

Figure 4.5 - Decellularization agents' container and the valve

While constructing

When the containers were designed, it was clear the necessity to open a hole on their wall to guarantee the connection with the valves. However, open a hole in the glass can cause fractures or fissures. For this reason, flasks of 2 liters (figure 4.6) were decided be used and two holes were made on their plastic cap (figure 4.7): one to allow tube connection and the other for filter assembly.

Figure 4.7 - – Flask of 2 liters from Schotts

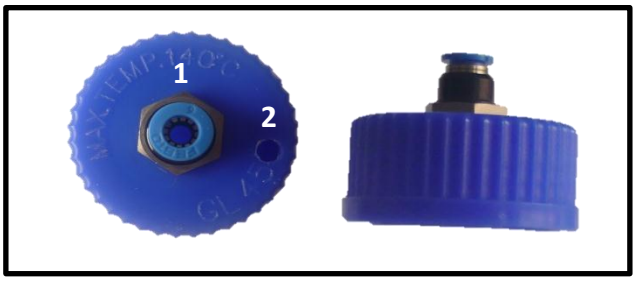


Figure 4.6- Cap of the flasks with two holes.

Hole 1 establishes the connection between the inside and outside of the container. Thus, an accessory screw (figure 4.7) was placed in this hole, allowing the tube to contact with the fluid and simultaneously providing isolation. On the other hand, in hole 2 was placed an air filter due to the necessity to contact with the atmosphere. If the filter would not be considered, the container could create vacuum pressure, leading to an inefficient process.

Moreover, as mentioned before, the diameter of the tubes can vary depending on the flow rate. In a first approach, the cap was thought to have different holes that could fit to different accessories screw. Thus, the same cap could be used for different tube diameters. However, due to the dimensions of the cap and the accessory screw, it was just possible to put one of it for each cap. Hence, each of the flasks has more than one cap. The cap will be selected according to the flow rate required.

Valves (10)

Valves regulate the flow of decellularization agents by opening or closing.

While designing

After leaving the decellularization agent container the fluid will pass through a valve. This controls the exit of the fluid from the decellularization agent container and dictates the entrance of the fluid in the perfusion chamber. If the valve is open, the fluid experiences the action of the peristaltic pump; if the valve is closed no additional fluid will be injected. As previously mentioned, the valve will be supplied by the relay card.

While constructing

The choice of the valves was mainly dependent of one factor: their biocompatibility with the decellularization agent containers. Thus, two way solenoid valves, G ¼, 24 Vdc from Bürkert, were selected, regarding to their compatibility for aggressive fluids and the follow main specifications:

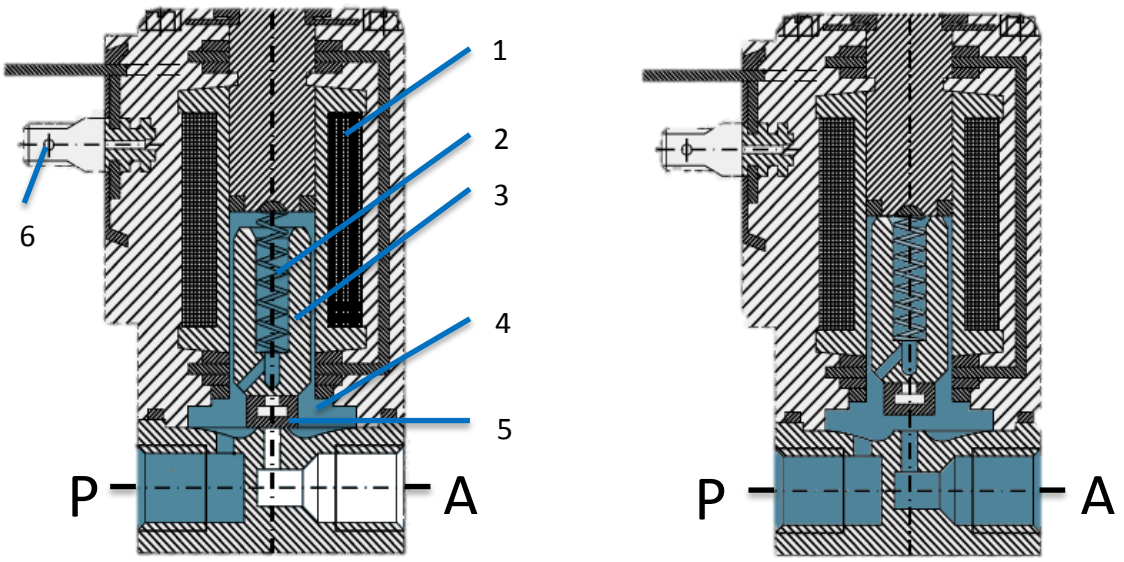
Body material: Polypropylene
Maximum temperature (ºC): 80
Maximum operating pressure (bar): 3
Operation: Direct Acting
Port Connections: G 1/4
Supply Voltage: 24 Vdc

Solenoid valves are control units which, when electrically energized or de-energized, either shut off or allow fluid flow. The actuator takes the form of an electromagnet. When energized, a magnetic field builds up which pulls a plunger or pivoted armature against the action of a spring. When de-energized, the plunger or pivoted armature is returned to its original position by the spring action.

For better understanding is possible to describe the function of these valves based on figure 4.8. Firstly, according to the mode of actuation, a distinction is made between direct-acting valves, internally piloted valves, and externally piloted valves. A further distinguishing feature is the number of port connections or the number of flow paths ("ways").

The selected valves are direct-acting two-way valves. Through-way valve, are shut-off valves with two ports: one inlet port (P) and one outlet port (A). When P is connected to the fluid, in the de-energized state, the core spring (2), assisted by the fluid pressure, forces the plunger (3) with the valve seal (4) onto the valve seat (5). Passage to outlet A is thus shutted-off. On the other hand, if the voltage

is applied, the solenoid core (1), with the valve seal (4) is pulled into the coil as the result of the magnetic force and the valve opens. The passage is unobstructed allowing fluid flow. Additionally, the solenoid valve is supplied by the relay card, using the electrical connection.



Legend:

1 – Solenoid coil	4 – Valve seal	P – Inlet port
2 – Core spring	5 – Valve seat	A – Outlet port
3 – Core, plunger	6 – Electrical connection	

Figure 4.8 – Direct-acting 2/2 way plunger type solenoid valve, shown closed at left and open at right. (Adapted from [www.burkertvalves.ca])

Perfusion chamber (12)

While designing

The decellularization cell was design based on the perfusion technique. Therefore, a versatile chamber was created, and their characteristics are shown in figure 4.9. The features and functions of each component can be followed below.

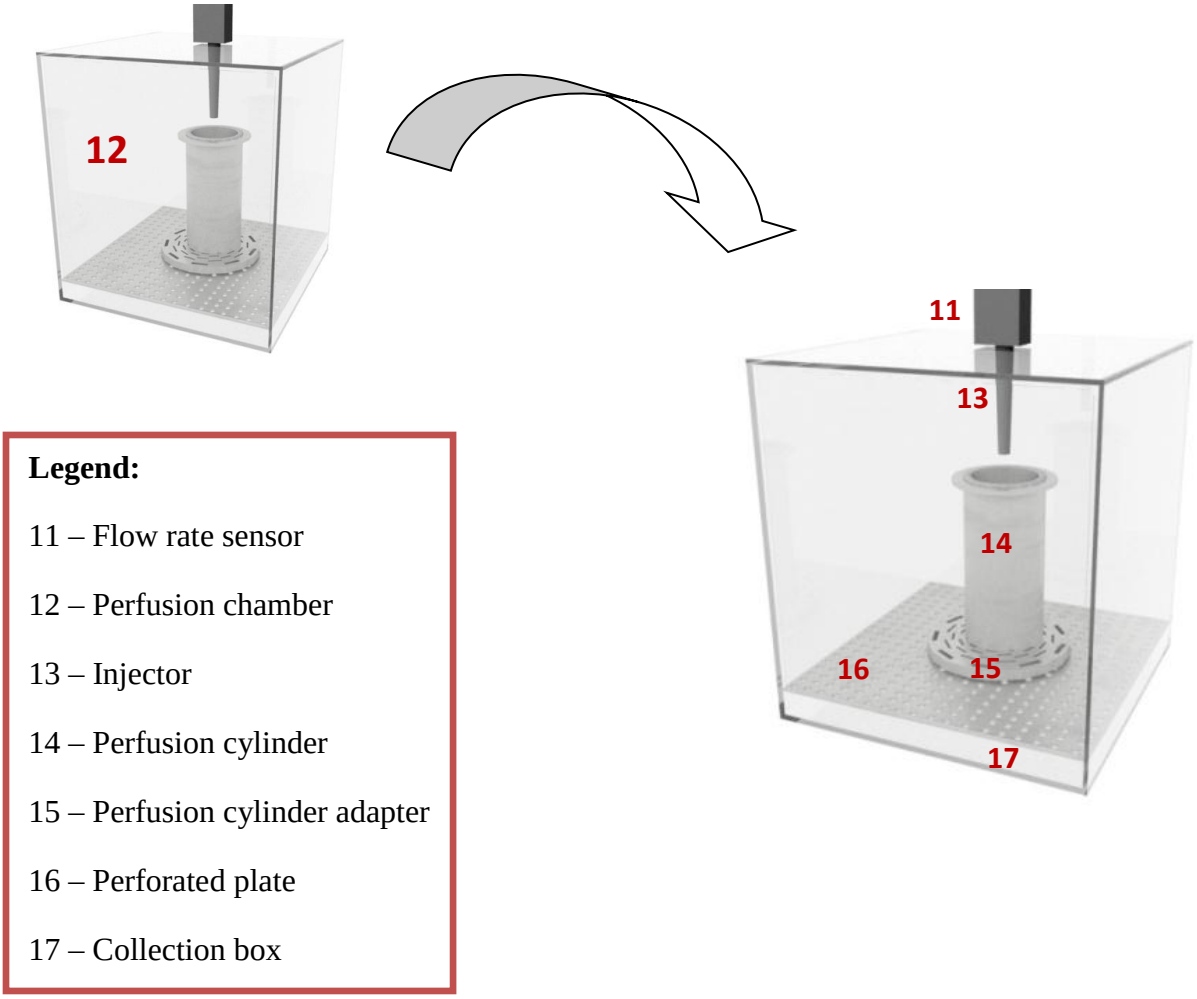
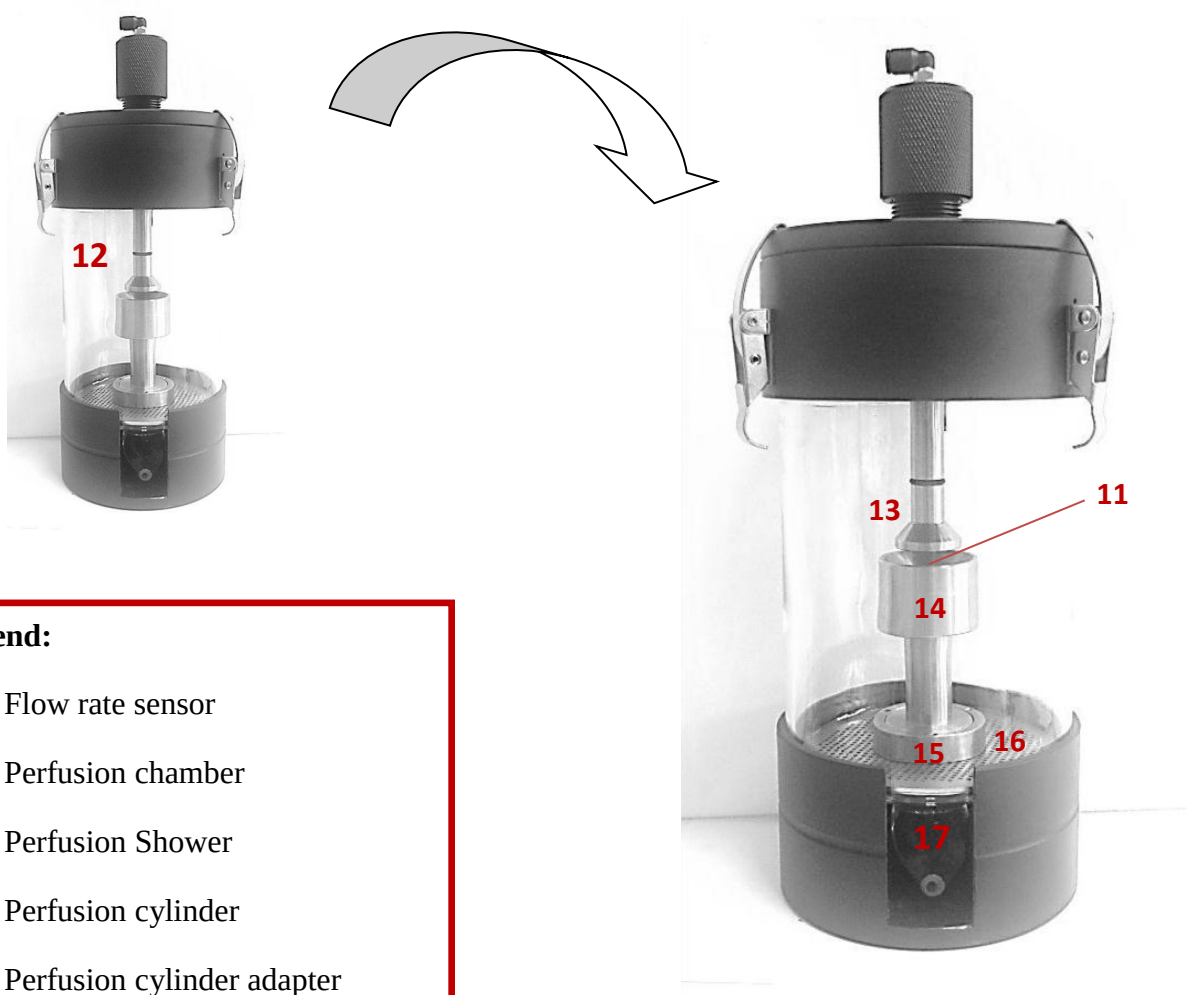


Figure 4.9 – Perfusion chamber and its components (design)

While Constructing

The decellularization cell was design based on the perfusion technique. Due to the characteristics of the perfusion chamber and their important functionality, their components were the most modified. As expected, a versatile chamber was created, and their characteristics are shown in figure 4.10. In their whole structure, the decellularization chamber was constructed in glass, plastic and stainless steel materials. When the decellularization agents might directly contact with the chamber, glass and stainless steel are required due to their biocompatibility and resistance. Otherwise, plastic (in black) can be a less expensive option, and thereby selected for the construction of the other elements.

Components 13, 14, 15 and 17 were integrally constructed in stainless steel due to their clear advantages. Stainless steel provides resistance to corrosion and staining, requiring reduced maintenance and presenting low cost. The most important: the biocompatibility of this material with decellularization agents and cellular material.



Legend:

11 – Flow rate sensor

12 – Perfusion chamber

13 – Perfusion Shower

14 – Perfusion cylinder

15 – Perfusion cylinder adapter

16 – Perforated plate

17 – Collection box

Figure 4.10 – Perfusion chamber and its components (construction)

Injector (13)

While Designing

The decellularization cell will use a needle as a structure to inject the decellularization liquid agent through the tissue. The apical extremity of the injector will be connected to the tube where the fluid passes through and therefore, the apical diameter of the injector will be dependent of the inner diameter of the tube. On the other hand, the diameter of the injector' tip should have the more convenient size for a particularly decellularization process. Thus, different injectors were considered to be built, depending on the distinct tube diameter, which in turn depend on the peristaltic pump, decellularization agents and decellularization process conditions.

The distance between the tissue placed horizontally in the apical extremity of the cylinder and the tip of the injector is also to be considered. The decellularization cell was designed considering a regulated distance by vertical motion of the injector position. Therefore, after construction, efforts could be made to optimize the protocol, and a relation between the distance and the impact of it in the tissue would need to be measured.

While Constructing

In a first approach, application of the decellularization liquid agent through the tissue considered the use of an injector, in which the inner diameter would be smaller than the diameter of the tissue to perfuse. Thus, the decellularization would be done accordingly to figure 4.11.

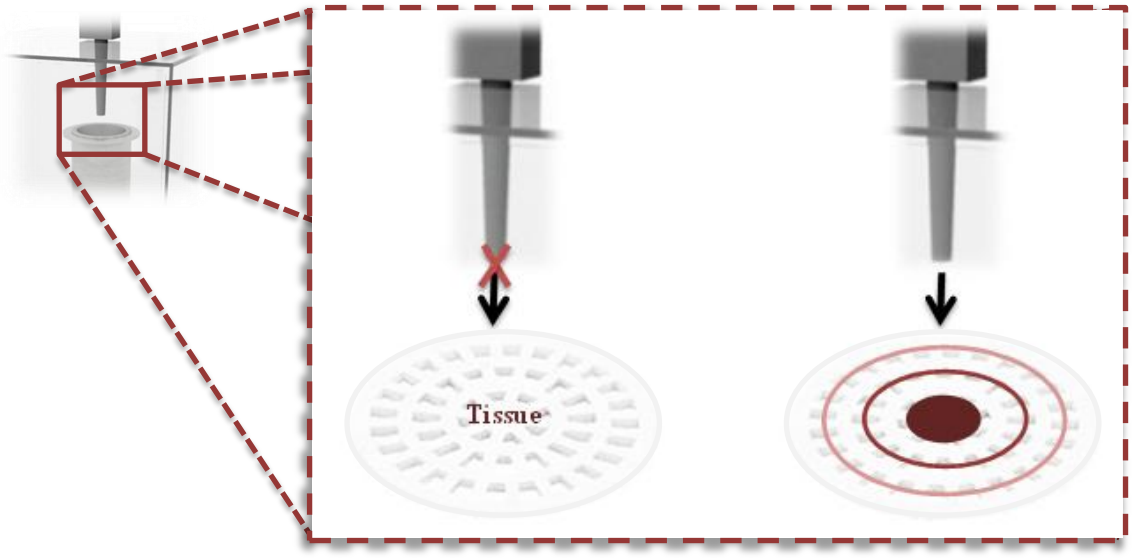


Figure 4.11 – Perfusion injector as it was initially designed. The fluid creates a heterogenic pressure field in the tissue. The central part of the tissue is forced to higher pressures than the peripheral part.

By interpretation figure 4.11, when the decellularization agent perfuses the tissue, it might create a heterogenic pressure field that is highest in the central part of the exposed tissue. When the distance to the center increases, the pressure created by the fluid on the tissue, decreases. Therefore, the decellularization of the tissue might vary leading to and inconsistently removal of cells. To circumvent this, a perfusion shower was created (figure 4.12).

Considering figure 4.12, the lid of the decellularization chamber was constructed as an integrated system in which the perfusion shower can be attached (A, B). As mentioned before, one of the requisites of the DecCell is to be a versatile system that allows removal of cells in tissues of different areas and thicknesses. Thus, different perfusion showers of distinct diameters had to be built. Thereby, the apical extremity has the same diameter for whole the perfusion showers (I), whereas the inferior parte, with the perforated shower (II), has different diameters.

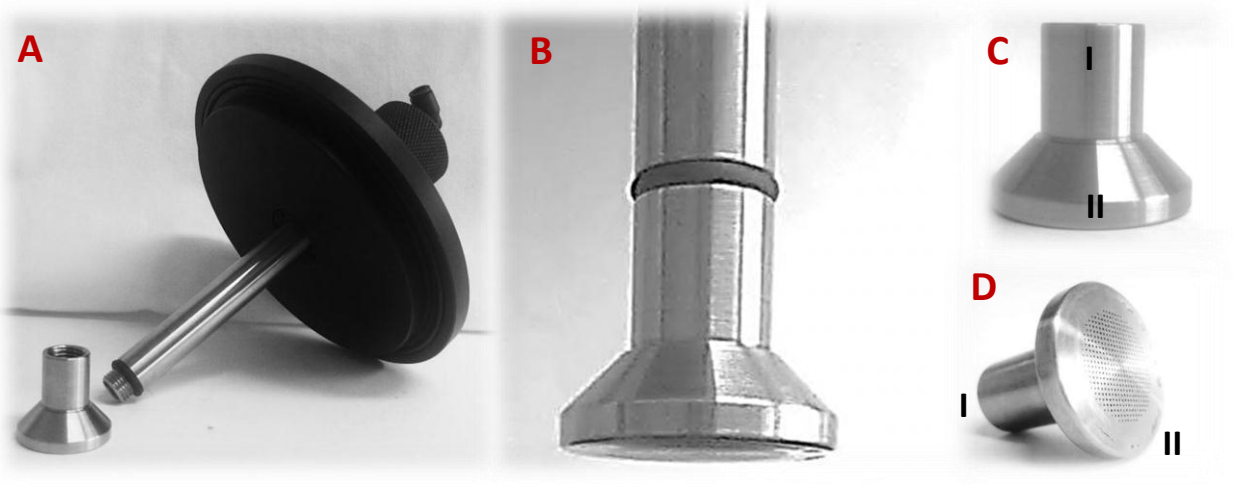


Figure 4.12 - Perfusion shower. (A) and (B) Perfusion chamber lid and its connection to the perfusion shower; (C) and (D) Perfusion shower in different views

Perfusion cylinder (14)

The perfusion cylinder serves as a structure where the tissue will be placed and immobilized.

While Designing

During the design of the decellularization cell a versatile system with different cylinder diameters, and adaptable to the distinct tissue diameters, was conceived (figure 4.13). The tissue can be placed horizontally on n the apical extremity of the cylinder, which is covered by a silicone surface in order to

maintain the integrity of the sample. As soon as the tissue is placed in the correct position, an O-ring can fix the sample, as shown in figure 4.14. The cylinders were designed in stainless steel due to their clear advantages. Stainless steel provides resistance to corrosion and staining, requiring low maintenance and presenting low cost. The most important characteristic is the biocompatibility of this material with the decellularization agents and the cellular material.

Figure 4.13 - Perfusion cylinders with various diameters

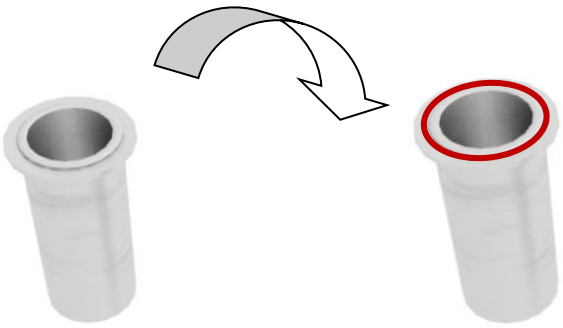


Figure 4.14 - Perfusion Cylinders and O-ring (red) to fix the sample.

While Constructing

The tissue can be placed horizontally in the apical extremity of the cylinder (figure 4.15-A.I). If necessary, a thin porous silicone net could be also placed under the tissue, to sustain the pressure caused by the fluid. Hence, as soon as the tissue is placed in the correct position, an O-ring can fix the sample, as evidenced by in figure 4.15-C.

Therefore, component B is attached to C as showed by the transition from the state D to E (figure 4.15). It is known that besides the area of the tissue, the samples can also have different thicknesses. For this reason, the perfusion cylinder provides an adaptive system, in which the component B can be more

or less twisted to C. Afterwards, the fluid release using the shower, will create a "fluid pool" in the upper part of the perfusion cylinder, which will force be fluid to perfuse the tissue.

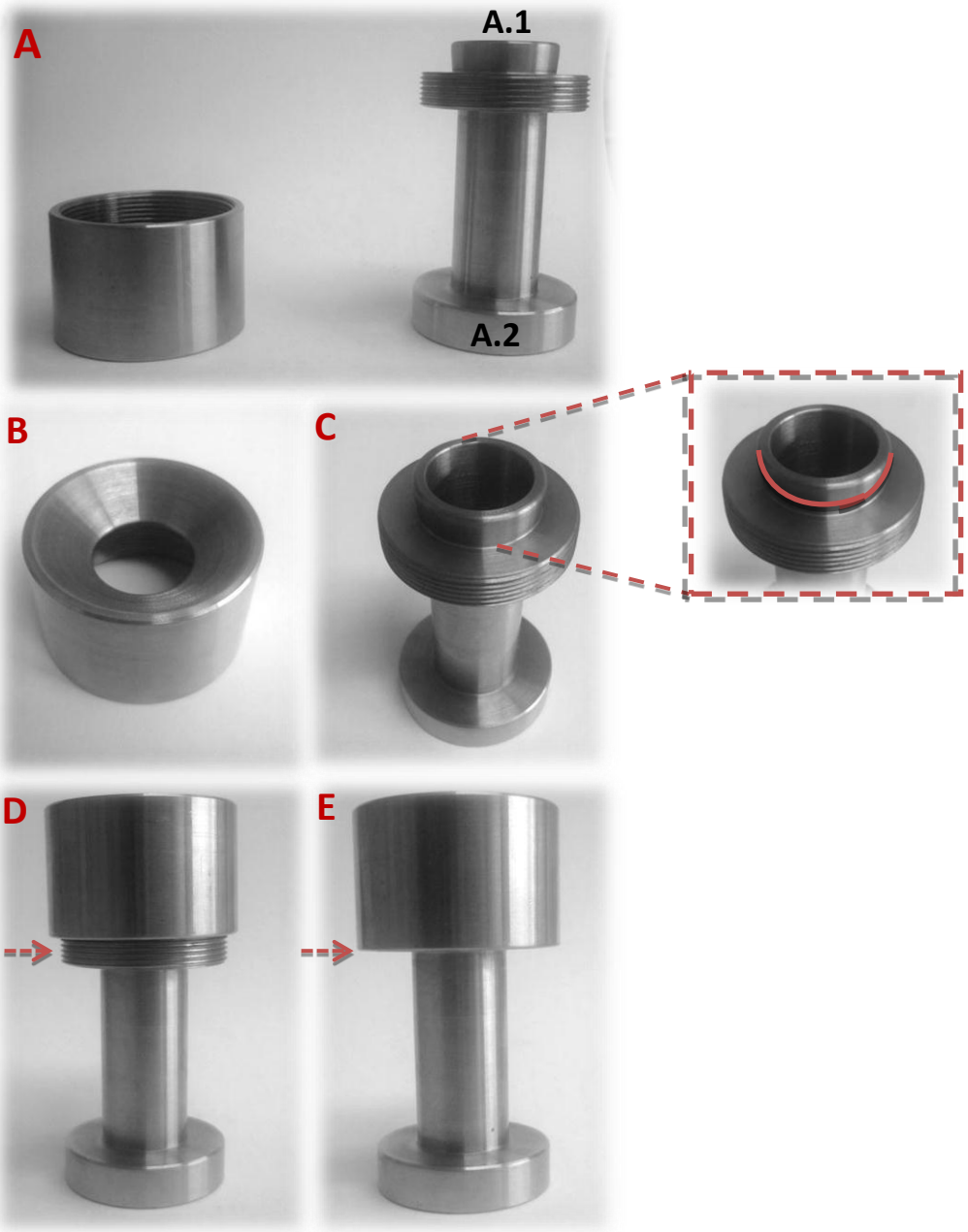


Figure 4.15 – Perfusion cylinder. (A) The two components of the perfusion cylinder (cap and cylinder); (B) The cap of the perfusion cylinder; (C) The cylinder and the localization of the O-ring; (D) and (E) The two components of the perfusion cylinder twisted.

Additionally, the distance between the tissue placed horizontally and the inferior part of the shower is also very important. Thus, the decellularization cell provides a regulated distance by moving the maniple showed in figure 4.16. Afterwards, efforts can be made to optimize the protocol, and a relation between the distance and the impact of it in the tissue can be measured.

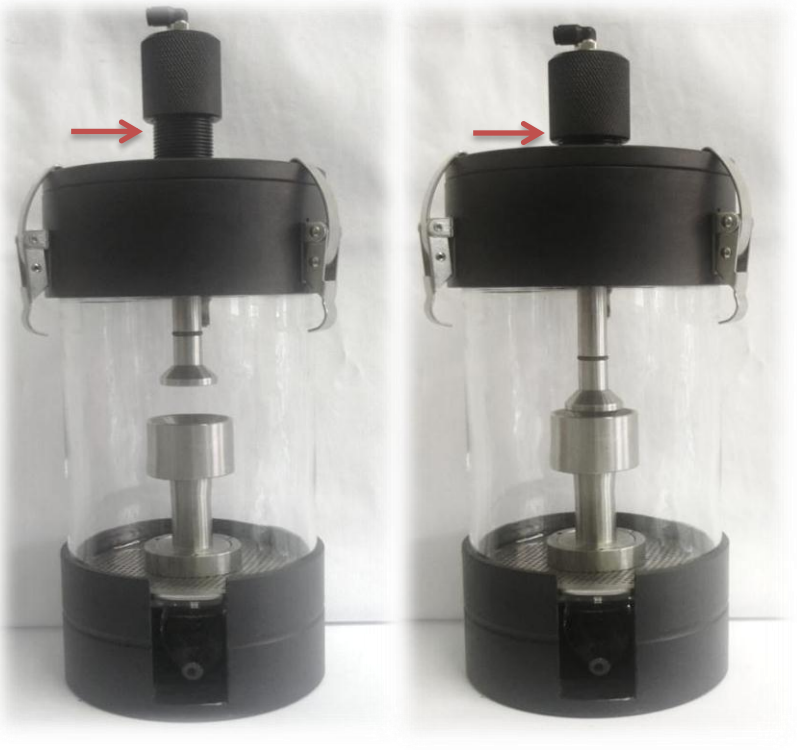


Figure 4.16 – The perfusion cell and the movement of the maniple

Perfusion cylinder adapter (15)

The perfusion cylinder adapter functions as a support to the perfusion cylinders.

While Designing

The required versatility of the decellularization cell to respond to different sample diameters leads to the design of different cylinders. Consequently, it was necessary to design a cylinder adapter that would function as a support for the wide range of cylinders. In figure 4.18 is possible to observe the whole cylinder adapter, an adapter for the most small diameter size. In other hand, by removing the inside

individual circular section of the whole adapter is possible to create a "new" adapter for another cylinder. Thus, each cylinder would have its correspondent adapter (figure 4.17).

The adapter was conceived in stainless steel due to the previously mentioned biocompatibility reasons. A particular characteristic of this adapter is the perforated structure of each individual circular section, in order to allow the drain of the fluid to the collection box. Additionally a radial inclination along the surface of the adapter was considered to facilitate fluid drain until reach the collection box.

Figure 4.18 - The whole structure of the perfusion adapter

Figure 4.17 - The perfusion adaptor divided by individual circular sections

While Constructing

Since the down part of the perfusion cylinder (figure 4.15-AII) has always the same dimensions, the same adapter can be used by all the different cylinders. This adapter is currently fixed to the perforated plate, as shows figure 4.20. Due to their small dimensions, the adapter did not need to be perforated.

Perforated plate (16)

The perforated plate provides support to the cylinder adapter and saves the surface of the bench where the decellularization process takes place.

While Designing

As shown in figure 4.9, the perforated plate is fixed above the collection box. Because the fluid is removed by suction from the collection box, the whole chamber can experience the pressure created by the vacuum. Since this might damage the integrity of the sample, the perforated plate can highly

minimize these effects while allowing the access of the fluid to the collection box. Figure 4.19 shows the perforated plate, design in stainless steel, in detail.

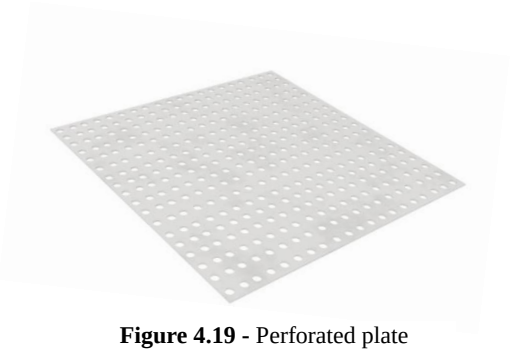


Figure 4.19 - Perforated plate

While constructing

The perforated plate (figure 4.20-A) was constructed according to their design. This plate is also well perforated, allowing the access of the fluid to the collection box.

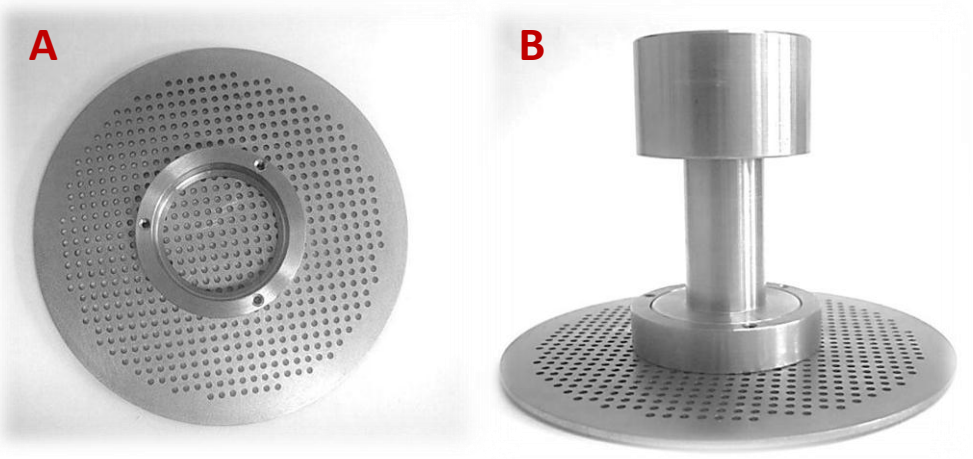


Figure 4.20 – (A) Perforated plate and perfusion cylinder adapter; (B) Perfusion cylinder connected to its adapter.

Collection box (17)

While designing

The fluid resulting by the perfused decellularization is collected in a box placed in the bottom of the perfusion chamber, as shown in figure 4.9. Thus, this fluid can be removed to the vacuum bottle that provides a local waste. The suction of the liquid is made adjacently to the perfusion chamber in order to

reduce the effects of this pressure, in the sample. Additionally, the floor of the collection box is thicker in one of the sides, proving an inclination that ensures that any fluid stays in the collection box.

While constructing

The fluid resulting by the perfused decellularization is collected in a box placed in the bottom of the perfusion chamber, as shown in figure 4.21-A. This fluid will be removed by suction to the vacuum bottle, which provides a local waste. As a particular characteristic, the collector has a conical shape, converging to the e point that connects with the vacuum bottle, facilitating fluid collection.

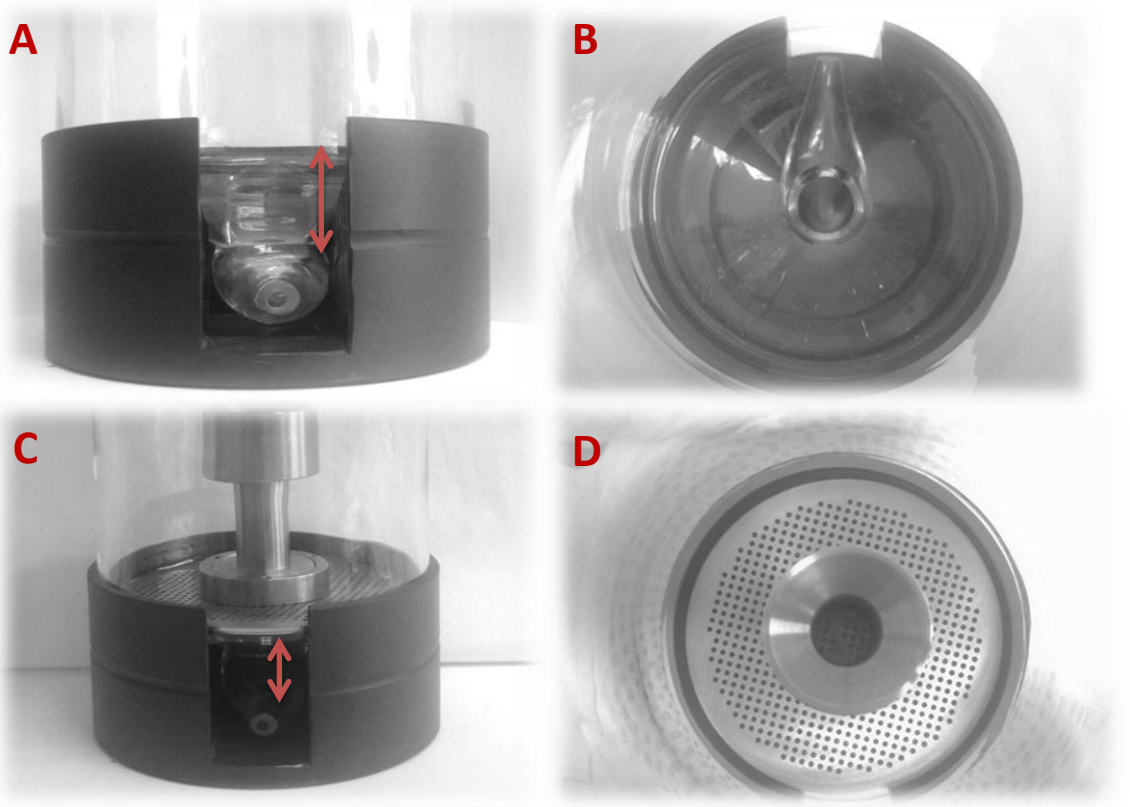


Figure 4.21 – Collection box.

Flow rate sensor (11)

A flow rate sensor is a device for sensing the rate of the fluid flow to control and guarantee that the flow rate has a desired value.

While designing

The flow rate sensor is supplied by the relay card and interacts indirectly with the peristaltic pump. If the valves are open, the fluid can start to flow and the flow rate must be controlled. When the sensor measured that flow rate is under or above the previously defined value, the peristaltic pump can act, readjusting the flow rate to its correct value. The sensor will be located before the perfusion chamber as showed in figure 4.9.

While constructing

If the sensor would be place before the perfusion chamber (figure 4.9), this device could just guarantee that the peristaltic was pumping the correct flow rate. However, due to the new characteristic of the perfusion chamber and their components, this sensor needed to be located in the upper part of the perfusion cylinder (figure 4.15 A), working as a level sensor.

When the fluid is moving from the perfusion shower to the tissue, it might create a "pool" of fluid, due to the different pressures. In this moment, the pumping does not have effect in the fluid anymore and the perfusion occurs due to the gravity and the suction effect of the vacusafe. Thus, is important to control the level of this pool:

- It cannot be higher than a certain level: it can cause float and damage the tissue due to the weight of the fluid;
- It cannot be less than a certain level: it could mean that the tissue is dry.

Therefore, a level sensor could be bought and the minimum and maximum levels would be defined. However, the several level sensors founded could not guarantee the good performance of this mechanism due to the small height of this "pool" and consequently, the close proximity of both levels. For this reason, a sensor level was also designed. Figure 4.22 shows a simplistic view of this component.

Figure 4.22 illustrates electric wires which are connected to the relay card. The relay card works as a switch, i.e., as an electrical component that can break and electrical circuit, interrupting the current. In this case, the relay card is a simple on-off switch and therefore, just when the two terminals are connected together, the electricity is conducted.

The interaction of these wires (blue – grey and red – grey), in a liquid medium, can produce a short circuit, translated in an electricity conduction. Thus, a digital signal is sent to DAQ. Regarding to A), the level of the fluid could not reach the minimum level sensor and therefore, no signal is sent to the data

acquisition. On the other hand, in case of B) and C), due to the short circuit, this signal is sent and in the final, the software might read this signal.

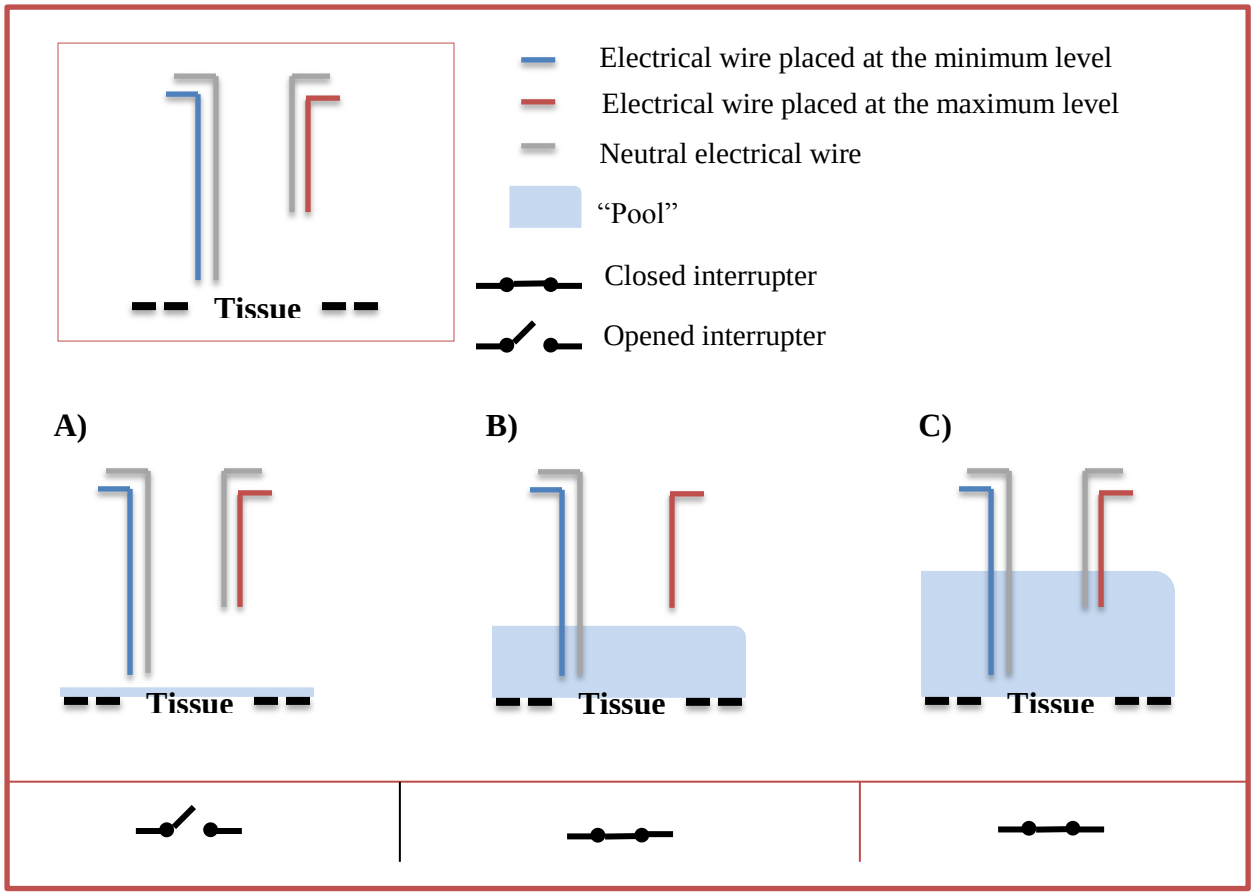


Figure 4.22 – Level sensor mechanism

Vacuum pump (7)

While designing

This pump provides a vacuum aspiration for the liquid waste disposal. The vacuum leads to a safe and convenient removal of the results of the decellularization process by perfusion. Additionally, a filter can be used in order to protect the unit from contamination and liquid entry. All components coming into contact with liquids can be autoclaved.

While constructing

The vacuum pump provides an aspiration system for the liquid safe disposal of liquids. Additionally, this structure should also provide the vacuum, assisting the perfusion of the tissue by the decellularization agent. For this reason, Vacusafe (figure 4.23) was bought, with follow main features:

- Hydrophobic filter for vacuum source protection;
- Shatterproof bottle for prevention of spills;
- Stand for securely holding the bottle in place;
- Autoclavability of the system components that contact liquid;
- Integrated pump with vacuum control;
- Self-closing connectors for avoiding escape of drops or aerosols from disconnected bottle and tubing;
- Level sensor detecting when the bottle is full and so preventing liquid overflow.

Thus, there are very clear their main advantages: Vacusafe protects from contamination, is an autoclavable system and has vacuum controlling, which also controls decellularization.

Figure 4.23 – Vacusafe from Integra

The decellularization cell is showed in figure 4.24. However, in this figure is not presented the tubes and the connection between the different components.

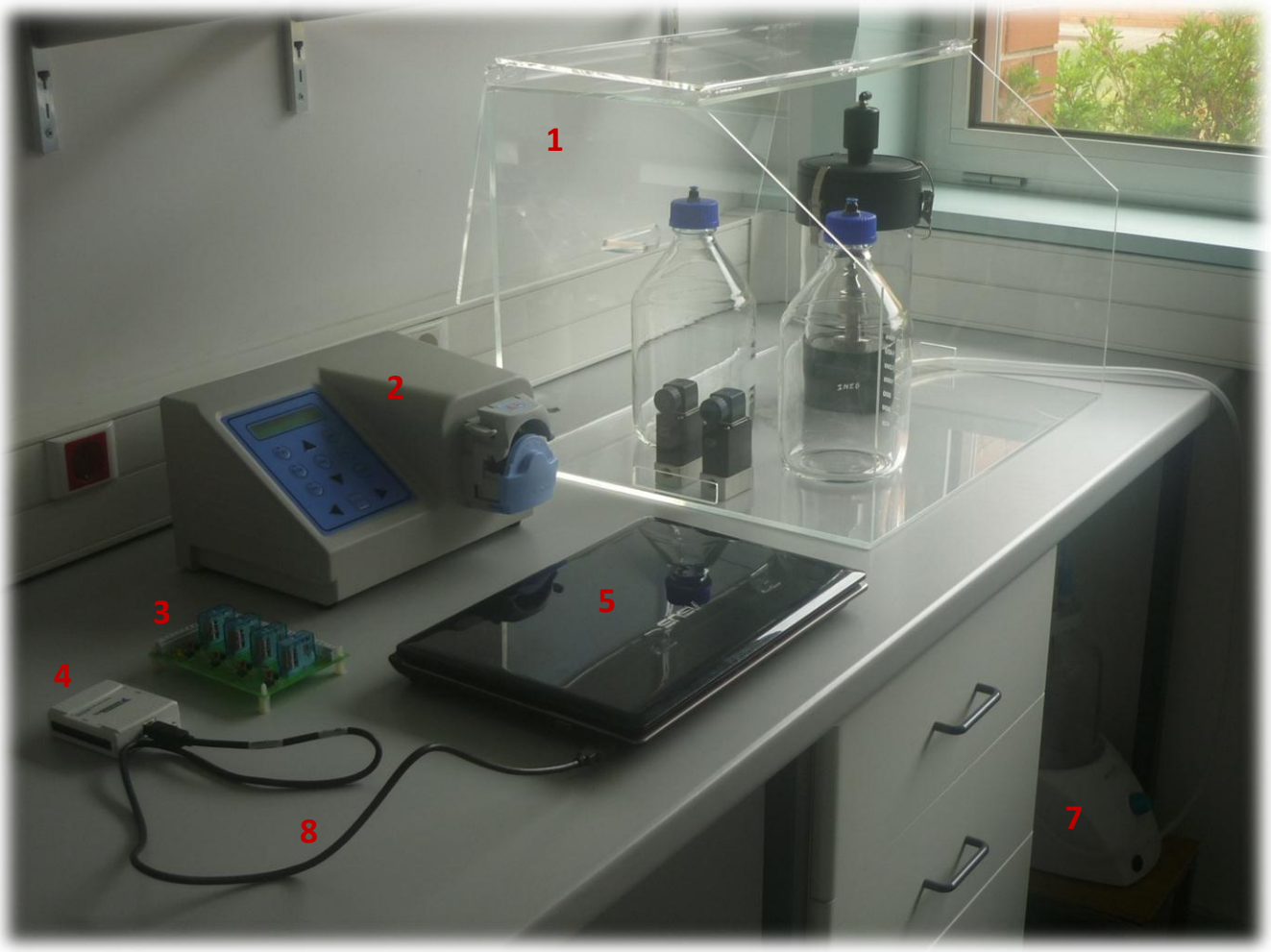


Figure 4.24 – The Decellularization Cell (construction)

Legend:

1 – Decellularization Chamber

2 – Peristaltic Pump

3 – Relay card

4 – Data Acquisition (DAQ)

5 – Computer

7 – Vacuum pump

8 – Connector Cables

Figure 4.25 – The decellularization chamber (construction)

Legend:

9 – Decellularization agent container

10 – Valve

12 – Perfusion chamber

4.3. Operating mode

The software installed on the computer (5) is able to control the whole system, by programmable control or manual control. Thus, by clicking on a button is possible to initiate the decellularization process. The valve (10) localized in the exit of the decellularization agent container (9) opens, allowing the fluid to leave the container. The peristaltic pump (2) induced by the software, can pump the fluid while controlling its flow rate by compressing the tube (6). Additionally, a flow rate sensor (11) is placed in the perfusing chamber (12), keeping the process under control, in terms of flow rate.

In the perfusion chamber, the fluid is injected, allowing liquid poured through the tissue. The waste results of the decellularization process can be collected (17) and sucked by a vacuum pump (7).

Since the decellularization cell is a dynamic process the tissue is always in contact with new fluid. After a specific fluid cycle it is possible to continue the process with another fluid by opening the valve of the second container and closing the first one. However, it might be necessary to clean the system between the two cycles. For example, the second container can work with PBS while the first container is being filled. The versatility of this constructed system provide several options that would have however to be tested in the future.

B. Decellularization Cell Program

DecCell was designed to be an automatic system. Thus, the user just needs to contact with the computer to control the process. The automation of the process is mainly divided in two paths:

1. Software (computer) → Valves
2. Level sensor → Software (computer) → Valves

1. Software (computer) → Valves

At first, the valve from a selected decellularization agent container needs to be open. As mentioned before, the valves of the decellularization cell are normally closed. Thus, the valves opens if a given voltage is applied, allowing fluid flow. Figure 4.26 describes this process.

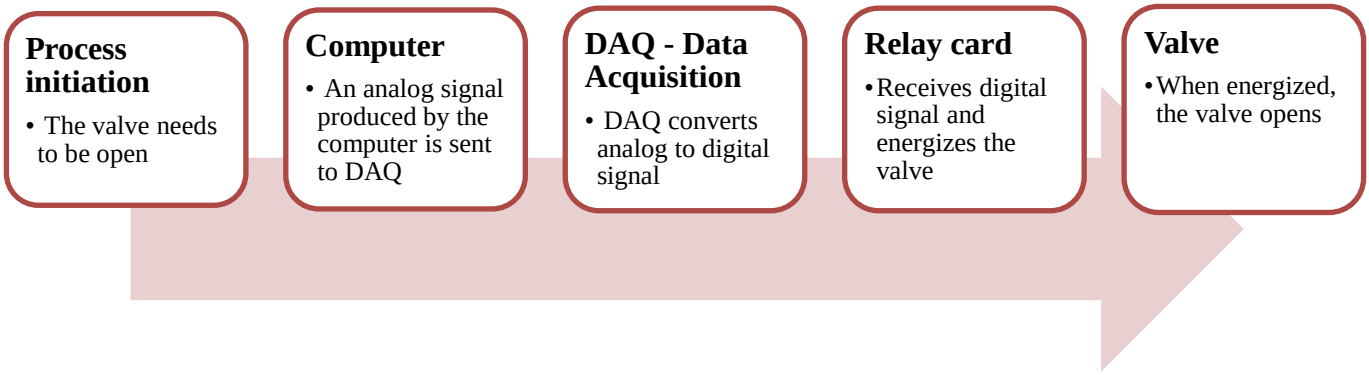


Figure 4.26 – Information flow from computer to the valve

2. Level sensor → Software (computer) → Valves

On the other hand, the flow rate of the decellularization liquid agent needs to be controlled. Thus, the level sensor is connected to the relay card (further details in the level sensor characteristics) which in turn sends a digital signal to the DAQ, which again controls the fluid rate defined at the peristaltic pump. The whole process can be followed in figure 4.27.

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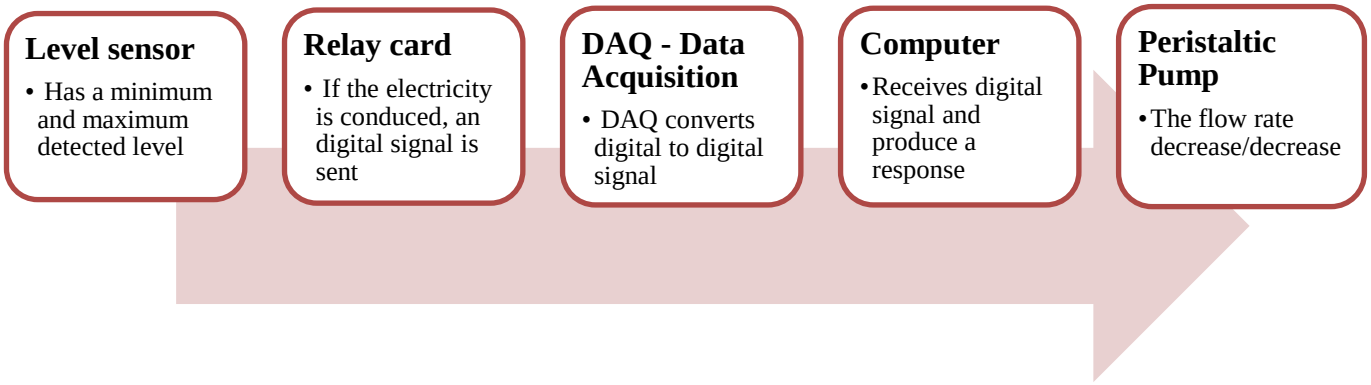


Figure 4.27 – Information flow from the level sensor to the computer

4.4. Automatic control

The decellularization cell provides an automatic control of the process since the user just needs to interact with the program in order to control the decellularization process.

At first, is important to define the main factors that are dependent of the flow rate:

- Tube inner diameter, since flow rate is higher with larger inner diameter;
- Length of the tube in the pump, measured from initial pinch point of the dispensing to the final release point. The flow rate is higher with longer length;
- The value of the RPM, since flow rate is higher with higher RPM.

These parameters must be identified in the program. However, since the tube inner diameter and the length of the tube in the pump are known before the decellularization experiment, the user just need to change the RPM values to increase or decreased the flow rate.

LabView® is a platform and development environment for a visual programming language from National Instruments. To initiate the programming language is necessary to have the communication protocol for the interaction of the LabView® and the peristaltic pump. Thus, command codes are inserted in the LabView® program to guarantee that peristaltic pump can read it and respond to it.

These messages from the software to the pump unit take the form:

<start of message> <command code> <[data]> <end of message>

Where,

<start of message> = ASCII[1] code 3A hex **(:)**

<command code> = ASCII code 41 to 50 hex **(A to P)**

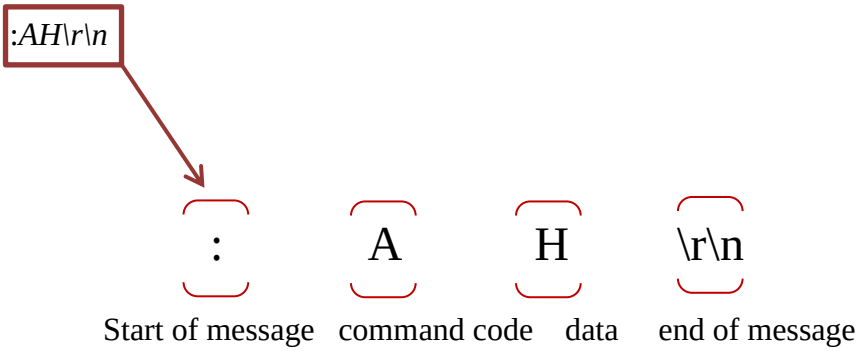
<[data]> = where a message requires data;

<end of message> = ASCII code 13 (Carriage return) **(\r\n)**

Table III shows the command set, including the command code and its meaning, the data and the reply from the peristaltic pump. As an example:

Message 1: *"The peristaltic pump must start to pump in counterclockwise (ccv) direction"*

Path: :*AH\r\n*



Message 2: *"Go to RPM setting"*

Path: :*DB\r\n*

[1] ASCII codes represent text in computers, communications equipment, and other devices that use text.

Table III – Table of command codes

Command Code	Function	Data	Reply from unit
A	INJECT KEYPRESS	Single character key code H = START CCW I = STOP J = START CW M = ENTER R = BATCH	None
B	READ SETUP	None	Setup data as follows: Control type (2 hex chars) Set RPM (2 hex chars) Selected tube (2 hex chars)
C	READ MOTOR CONTROL	None	Motor control (2 hex chars)
D	GO TO MODE	Single character mode code A = Normal mode B = RPM setting C = Tube setting D = Flow setting	None
E	WRITE SETUP	Setup data as follows: Control type (2 hex chars) [*1] Set RPM (2 hex chars) Selected tube (2 hex chars)	None
G	READ CALIBRATION	None	Expected volume (4 hex chars) Actual volume (4 hex chars) Both x 0.1 mL
H	WRITE CALIBRATION	Expected volume (4 hex chars) Actual volume (4 hex chars) Both x 0.1 mL	None
I	READ MEASURED RPM	None	RPM (2 hex chars)
J	READ RPM LIMITS	None	Minimum RPM (2 hex chars) Maximum RPM (2 hex chars)
K	LOOKUP FLOW RATE	RPM value (8 bit raw binary)	Flow rate (4 hex chars)

4.5. Manual and programmable control

The digital data received by the computer and the analog data that it sends, can be translated in a specific program which will automatically control the decellularization system. The interface between the programming language and the user is presented in figure 4.28. The program was divided in two main options: manual control and programmable control. In manual control the user can define the RPM value during the experiment. On the other hand, in programmable control, the values of RPM are already defined.

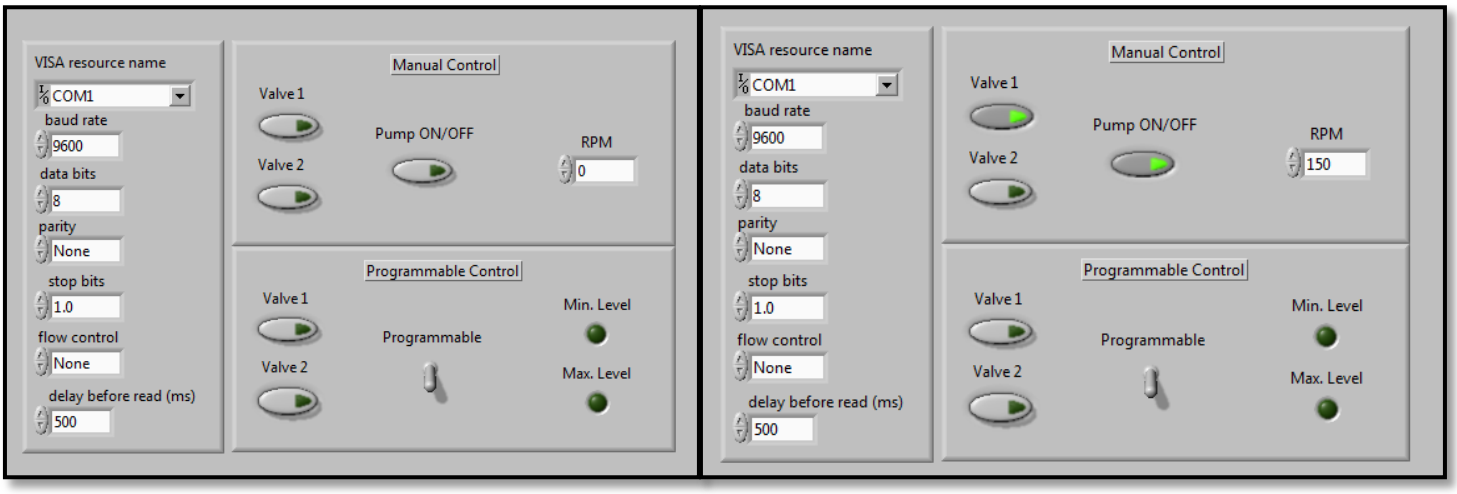


Figure 4.28 – Interface panel of the program

Regarding the manual control, one of the two valves can be selected by clicking in the respective button. The pump will be also activated and the value of RPM (rotations per minute) must be defined. In this case, the RPM could be decreased or increased by manually defining a new value. When the user wants to stop the decellularization process, the pump can be stopped by clicking on the button. The presence of the green light means that the valve is open and that the pump is on.

Programmable control the other alternative to control the opening of the valves and the flow rate. The interface panel (figure 4.28) is possible due to the creation of a program, in the LabView® platform, which follows the flowchart showed in figure 4.29, designed by us.

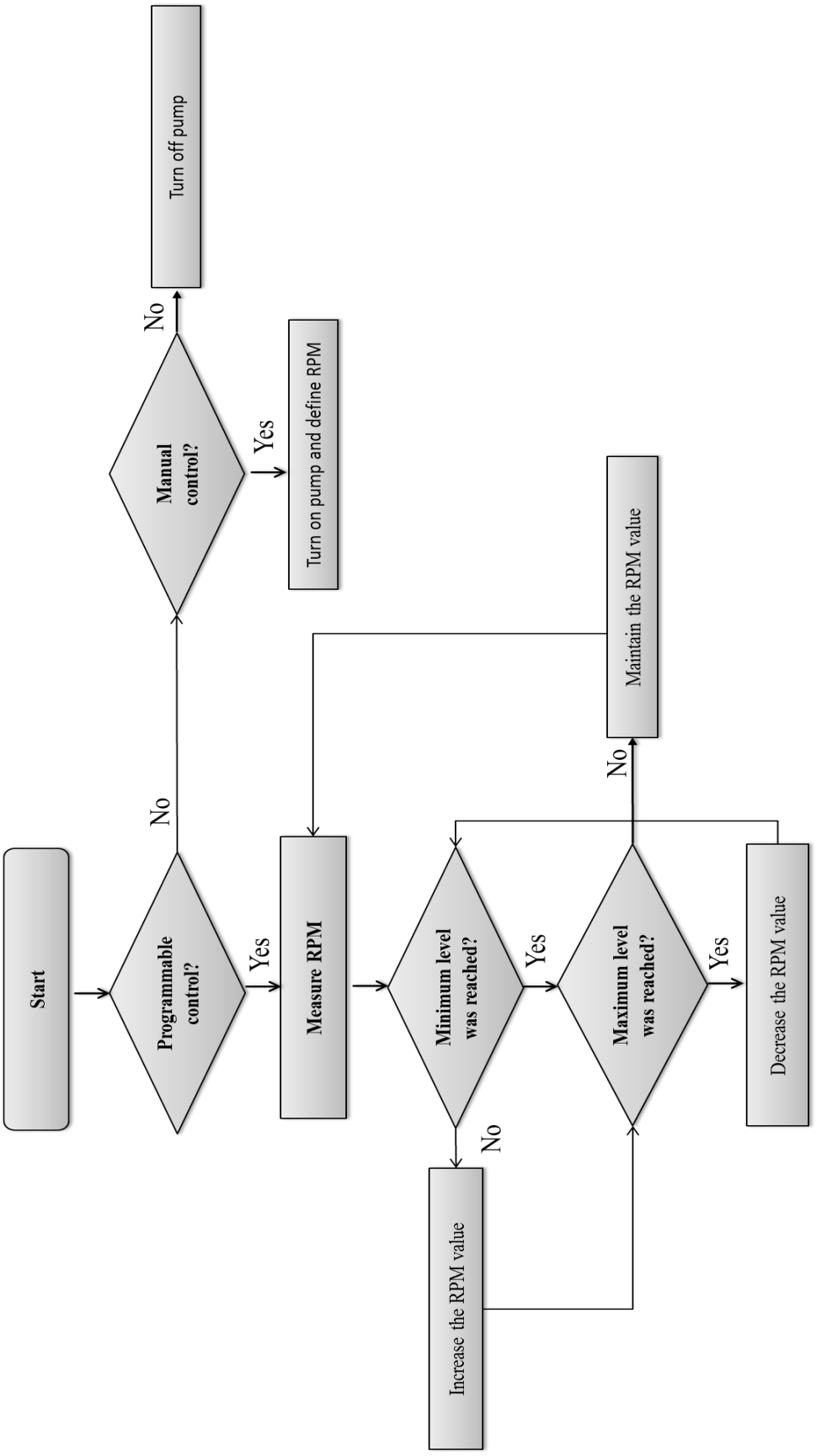


Figure 4.29 – Flowchart of the program that was created to automatically control the decellularization cell

This flowchart (figure 4.29) represents the data flow of the program. The LabView® block diagrams were created to insert the command codes which control the RPM, and consequently the flow rate of the decellularization agent. Figure 4.30 and 4.31 are examples of specific steps of this block diagrams.

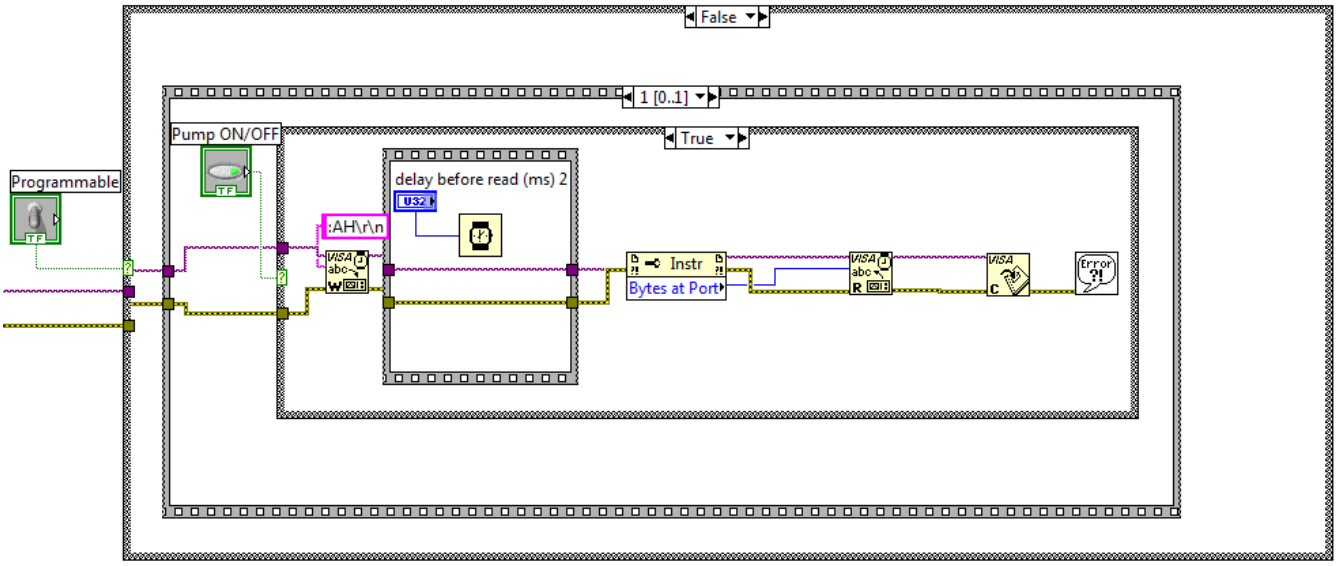


Figure 4.30 – Manual control.

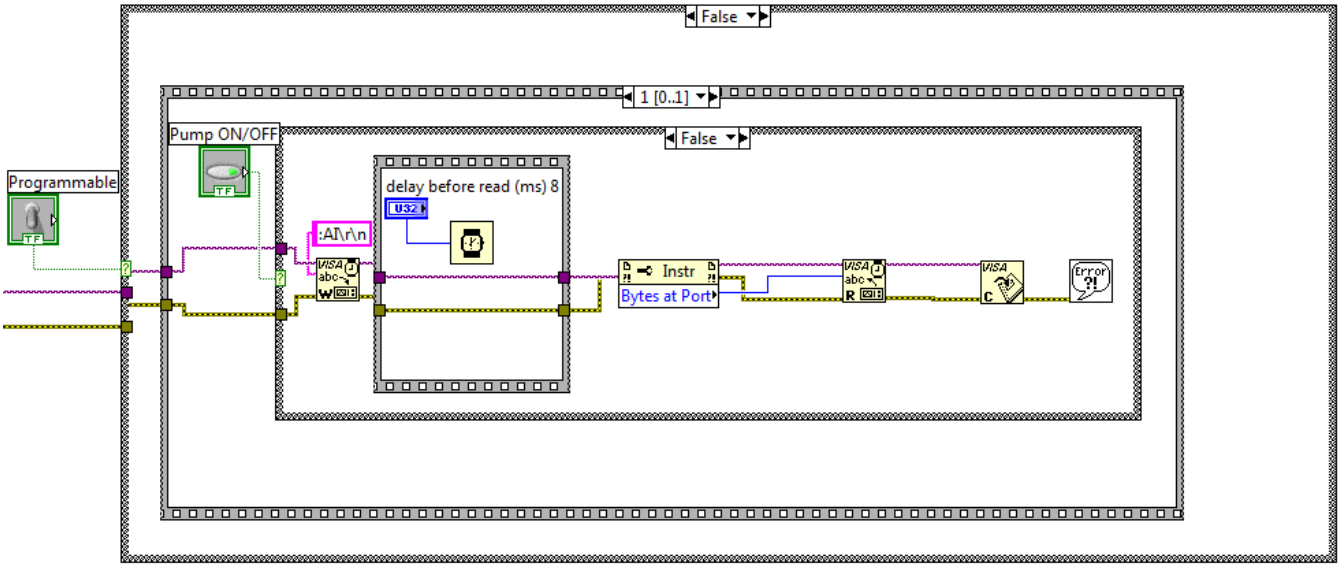


Figure 4.31 – No manual or programmable control

Through the interface represented in figure 4.28, the user is able to choose manual or programmable control. If manual control is chosen, the external box of the block diagram will assume the Boolean value false. The order of the decision steps can be compared to the order of the boxes, where the information flows from the external box to the internal, varying between the values false or true.

In manual control, the user can choose to turn on (Boolean value true) – figure 4.30 – or off (Boolean value false) – figure 4.31 – the pump. In case of figure 4.30, the command code permits the start of the pump function by acquiring the value of "AH". Although, if it assumes the value "AI", leads to the turn off of the pump. Other command codes, not showed in the figures, can be inserted to specify the value of RPM.

On the other hand, if the programmable control is selected, the program will check if the level sensor has detected the minimum flow rate. If not, a message is sent for the pump to continue working (:AH) and to increase the RPM value. As it can be seen in the figures 4.32 and 4.33, our system has a binary variable (0 or 1) whose value is alternatively being changed in each cycle. The value 1 corresponds to the command code AH and the value 0 corresponds to the command code "value RPM". The latter represents the limit value until when the RPM can be increased and it will be defined later on after future experiments.

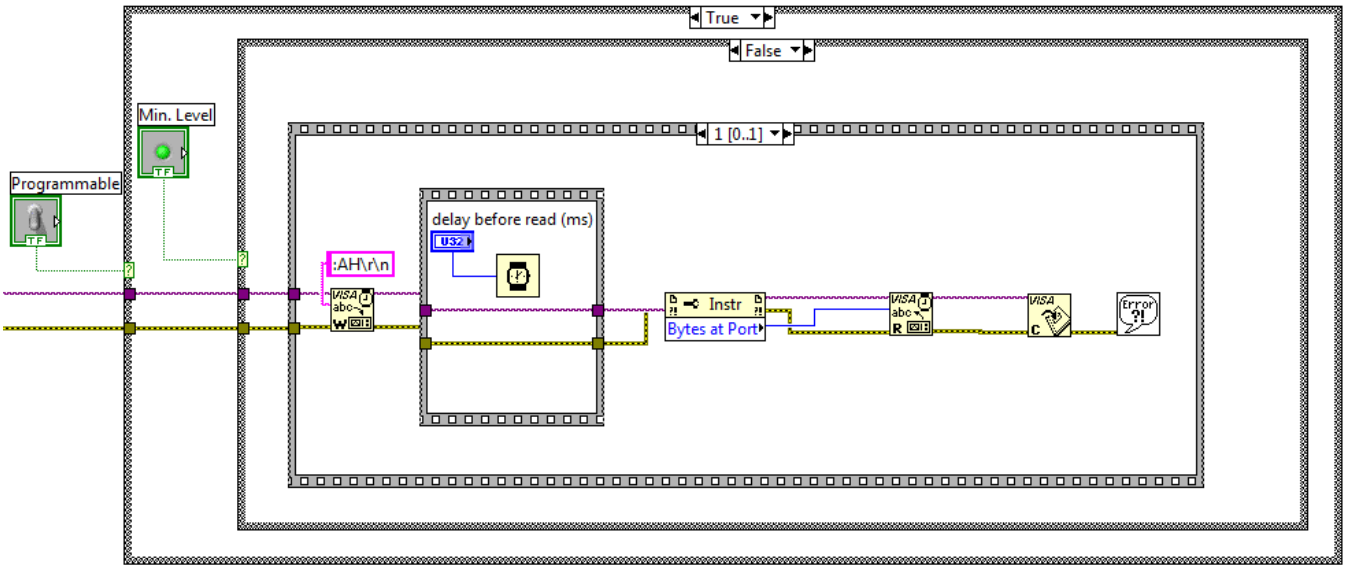


Figure 4.32 – Programmable control when the minimum level was not reached (1)

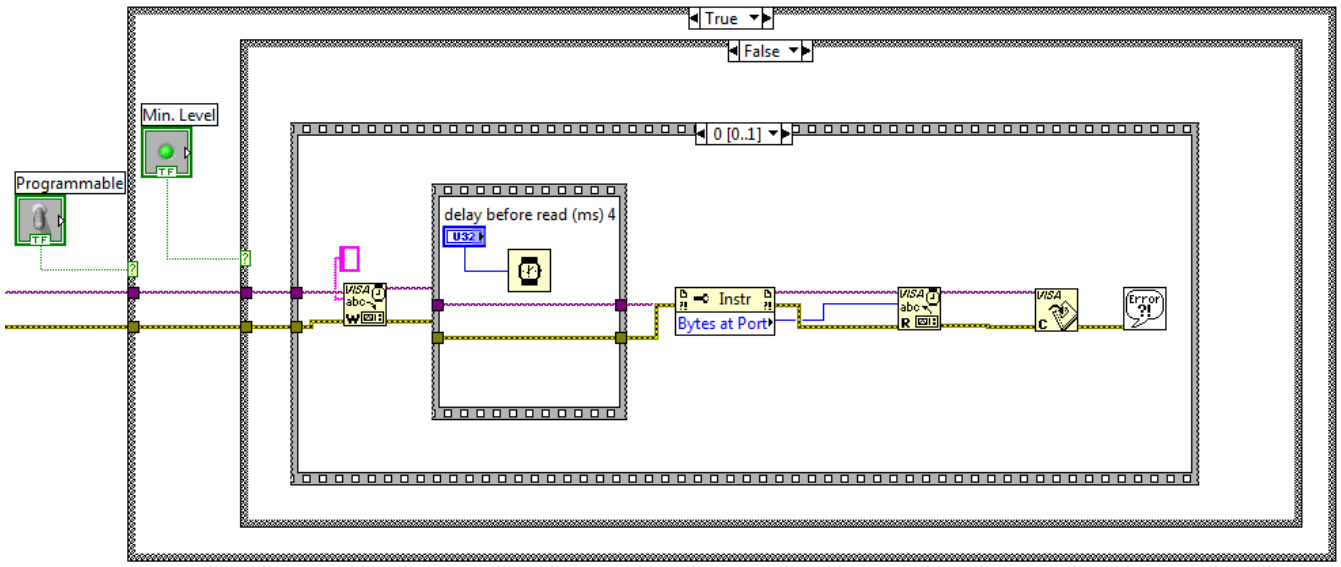


Figure 4.33 - Programmable control when the minimum level was not reached (2)

Likewise, the system also checks if the level has reached a value between the minimum and the maximum. If so, then the RPM value is not altered. When the maximum level is achieved, the system sends a message to the pump, this time to lower the flow rate (rather than increase).

The manual control will be very useful to perform optimization of the protocols. This is because the user can attempt to input different values of the flow rate. On the other hand, when the ideal flow rate is reached, for one specific experiment, the user can benefit from the programmable control.

5. Conclusions

Tissue engineering and regenerative medicine have been rapidly developed as an encouragement to understand processes of our body, cures for several diseases or even to "create life" by restoring or replacing tissues or organs through the use of biomaterial scaffolds. The extracellular matrix, as a complex structural entity that surrounds and supports cells, can be essential on cell behavior as migration, adhesion, survival and differentiation, due to the important interactions between cells and ECM. This background aroused our interest in developing decellularization of ECM, e.g. creating an innovative matrix based in human ECM which can give us insights regarding ECM-cell interactions.

This powerful potential of ECM has created great enthusiasm and led us to design and construct a decellularization machine dedicated to a successful removal of cells while maintaining the mechanical, chemical and structural properties of the ECM. We think that this is an innovative approach as we did not find anything similar in the reviewed literature. The most common approaches about ECM machines are related to the repopulation of acellular matrices.

In order to support the design of DecCell, different programs were used, enabling the 2D drawings (ArchiCAD 14), the models (3D Studio Max Design 2011) and the renderings of these models (MentalRay). Afterwards, in order to construct our DecCell, some components were bought whereas others were custom-made. Moreover, we wanted our machine to be automatedand for this reason, we have designed a program in a platform for a visual programming language - LabView®.

In conclusion, we reached our goal since the DecCell was designed and constructed as a automated and versatile system, i.e., functional for different sizes and thicknesses of human tissues. This apparatus is also automatic and therefore, its experiments can be programmable, and might provide a dynamic perfusion system suitable for human matrices decellularization. As expected, due to the lack of time, we

could not validate our machine teting distinct decellularization protocols. Accordingly, in the near future the decellularization will be tested and human gastric and colon fragments obtained from cancer patients surgical resections will be decellularized. Different specimen sizes will be decellularized using ultrasounds followed by perfusion with ionic and/or non-ionic detergents.

To confirm that this product meets the needs of its users, different validation criteria will be used focused on the advantages that the decellularization cell might provide, namely, reduction of the exposure time to the decellularization agents, and also a dynamic decellularization and versatility by providing decellularization of different type of tissues with different area and thickness.

We believe that this decellularization system might be extremely useful for basic and applied research in the area of bioengineering and oncobiology but also to tackle fundamental questions related to ECM components and to their interaction with their cellular counterparts. This decellularization cell can be used by researchers or companies interested in studying the dynamic behavior of the extracellular matrix and also in transplantation or regeneration of tissues or organs due to their application as homo- or a heterograft.

6. Future Perspectives

Our next step emphasizes the validation of the decellularization apparatus, by confirming if this product meets our needs. Although the potential of the decellularization cell, it is essential to analyze that the decellularization is effective: it leads to an efficient removal of cells and nuclear material of a given tissue, while maintaining the biological activity, mechanical integrity and composition of the extracellular matrix.

We believe that this decellularization cell can be translated in essential advantages, such as:

- Effective decellularization;
- Less exposure time to the decellularization agents;
- Dynamic decellularization, i.e., the fluid is always in circulation;
- Versatility by providing decellularization of tissues with different area and thickness

Validation can be divided in three criteria:

1. Perfusion technique vs. immersion technique
2. Variation of parameters
3. Optimization of protocols

2.1.1. Perfusion technique vs. immersion technique

In a previous protocol of our group, immersion technique was tested and 6 or 10 µm cryosections of human matrix tissue were decellularized. This protocol used Hypotonic buffer and gentamicin for 18 hours, followed by 0,5 or 1% of SDS exposure for 1 day. At final, the tissue was treated with DNAse for 3 hours.

As a first approach, it will be useful to perform the decellularization by perfusion technique on the same conditions (detergent concentration, same tissue and exposure time). Thus, the results can be characterized and it will be possible to infer if the decellularization cell can be effective. However, even if this structure leads to a successful decellularization, any advantage was provided because the conditions were exactly the same.

Therefore, it is necessary to change some parameters, such as exposure time or concentration of the decellularization agent, and analyze the results. This validation criterion should respond to: "What parameters can be changed without affecting the effectiveness of the decellularization?"

Within this criterion, it is fundamental to evaluate cell removal. There are many methods to determine the efficiency of the removal of cellular material from tissues. For example, Woods et al used standard histological staining with Haematoxylin and Eosin as a first inspection to determine if nuclear structures could be observed, as well as Baiguera et al, in their experiments for decellularization of human trachea (Baiguera, Jungebluth et al. 2010), heart valve (Zhou, Fritze et al. 2010), and pericardial tissue (Mendoza-Novelo, Avila et al. 2011) and bladder acellular matrix (Brown, Brook-Allred et al. 2005).

Apart from DNA analysis, the quantification of DNA content can also be used as an indicator of the presence of residual cells in decellularized tissues. According to Mendoza-Novelo et al, the DNA from

either native or decellularized tissue samples of known masses can be isolated, purified and rehydrated per triplicate using the Wizard_ Genomic DNA Purification Kit (Promega) and calculated its concentration spectrometric measurements (Mendoza-Novelo, Avila et al. 2011). On the other hand, in the experiments of Lu et al, the biological scaffolds (native as control and decellularized) were digested with papain solution and the lysates were used for detection of the DNA amount (Lu, Hoshiba et al. 2011). As suggestions, the inspection of the presence of DNA can also be performed by fluorescent staining for labeling DNA, as 4', 6-diamidine-2-phenylindole (DAPI) (Kapuscinski and Yanagi 1979) or Hoechst (Kakkar, Suruchi et al. 2005). Additionally, assays with propidium iodide and PicoGreen have been developed to provide quantitative data regarding the presence of DNA within the sample (Kral, Widerak et al. 2005).

2.1.2. Variation of parameters

After considering that decellularization cell is effective in cell removal, further experiences can change other parameters, as size of the tissue, area and thickness, as well as the flow rate. The decellularization cell provides versatility due to the different cylinders where the tissue will be placed. Different models can be adopted regarding to a specific tissue:

- Same area, same flow rate, different thickness;
- Different area, same flow rate, same thickness;
- Same area and same thickness while variations of flow rate.

The first model is the most important since one of the major features of the decellularization by perfusion is to provide an effective removal of cells to thicker tissues, which is not observed for others techniques.

2.1.3. Optimization of protocols

The decellularization cell can represent an advantage if it will result in an optimization of protocols. The two criteria mentioned before are the first steps to reach this goal since they will provide new insights about the decellularization cell and how it can be more effective. For example, a reduction of

exposure time might lead to a minimization of changes on the ECM integrity. On the other hand, it can be also possible to reduce the concentration or quantity of the detergent, which might be less aggressive to the tissue.

Although, regarding to an optimization of protocols, all the scenarios must be considered and ultimately, each result needs to be characterized. As previously mentioned, an evaluation of the cell removal is essential to measure the success of the decellularization. However, it is also essential to confirm if desirable components of the ECM are retained, such as collagens, GAGs, adhesion proteins, etc.

For example, for decellularized bone–anterior cruciate ligament, Woods et al (Woods and Gratzer 2005) measured the collagen content by determination of hydroxyproline, according to the procedure of Woessner (Woessner 1961). In order to characterize collagen composition in the bladder wall prior to and following extraction, Brown et al analysed native and acellular bladder by using monoclonal antibodies to collagen types I, III, and VI (Brown, Brook-Allred et al. 2005). On the other hand, Lu et al determined collagen contens using the Sircol collagen assay (Biocolor) (Lu, Hoshiba et al. 2011).

Another major component of the ECM are GAGs and can also be used as an indicator of the alteration in structrural composition of decellularized tissue. Woods et al (Woods and Gratzer 2005) expose the samples to a solution containg papain to solubilize proteoglycans. After digestion, a dimethylmethylene blue (DMMB) dye-binding assay for sulphated GAGs, using chondroitin sulphate as a standard, was performed on the digested samples using the procedure outlined by Farndale et al (Farndale, Buttle et al. 1986). In another experiment, native and decellularized tissue samples were lyophilized, minced and weighted and also digested using papain solution (Mendoza-Novelo, Avila et al. 2011).

Some properties as surface topography, composition and general information, may also be given by some equipments. For examples, native and bladder acellular matrix were fixed in glutaraldehyde, frozen in liquid nitrogen and frezze fractured on cross-section in order to be examinated under Scanning Electron Microscope (Brown, Brook-Allred et al. 2005). This study could give insights about the integrity of the acellular matrix. The morphology and structural integrity of native and decellularized pericardial tissues were also observed by low-vacuum SEM (Mendoza-Novelo, Avila et al. 2011). For this, wet samples were frozen with liquid nitrogen and then examined by SEM. In their experiments to measure the effect of the different detergents in heart valves decellularization, Zhou et al used Two-photon Laser Scanning Microscopy (LSM). Thus, the obtaining of high-resolution optical images with

depth selectivity, allowed the characterization of the acellular tissue (Zhou, Fritze et al. 2010). As suggestion, other techniques can be used as Atomic Force Microscopy, Dynamic Mechanical Analysis (DMA) and High-resolution Confocal Microscopy.

As future perspectives, this decellularization cell might have a very positive impact on the quality of life. This construct can make possible the evolved of tissue regeneration by use an acellular matrix as a bio-scaffold, providing an elimination of negative host responses, after autologous repopulation. On the other hand, the study of the characteristics of ECM and its components is essential to understand very specific events that occur at the cellular level. The research may lead to new insights into the mechanism of metastasis, and consequently, to new therapeutic targets for cancer disease.

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