Politecnico di Milano
Scuola di Ingegneria Industriale e dell'Informazione
Corso di Laurea Magistrale in Ingegneria Biomedica

Vascularization of a scaffold made by hydrogel through a hydro-soluble vascular network obtained with a 3D FDM printer

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Anno Accademico 2017-2018

Ringraziamenti

Ringrazio la professoressa Sara Mantero per avermi dato la possibilitá di lavorare a questo progetto e avermi assistito durante la sua realizzazione.
Ringrazio le ragazze e i ragazzi del LaBS per la loro disponibilitá e simpatia, é stato bello lavorare a questa tesi in vostra compagnia e poterla realizzare anche con il vostro aiuto. Il clima che ho respirato in questo laboratorio é stato di amicizia e aiuto reciproco, siete riusciti a farmi sentire come in una seconda casa, per questo vi ringrazio molto. In particolare vorrei ringraziare: Monika, Luca, Gigi, Marco e Francesco che mi hanno aiutato a risolvere vari problemi e ad aggiungere preziosi elementi alla mia tesi.
Ringrazio le ragazze e i ragazzi del +lab per la disponibilitá e gli aiuti ricevuti per gestire al meglio le difficoltá che si possono incontrare durante l'utilizzo di una stampante 3D.
Ringrazio gli amici che mi hanno sostenuto ed aiutato in questi anni.
Ringrazio la mia famiglia che mi ha sostenuto durante il percorso di studi ed in particolare mia mamma perché: é una persona fenomenale che ha avuto la forza di starmi vicino soprattutto nei momenti piú difficili, di cui posso fidarmi ciecamente e se ora sono qui e ho finalmente concluso questo percorso di studi lo devo non solo agli aiuti economici ricevuti di cui comunque ringrazio molto ma soprattutto a lei per il sostegno morale.

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Introduzione

L'obbiettivo della medicina rigenerativa é quello di essere un'alternativa ai trapianti e alle protesi per rigenerare tessuti danneggiati o malati che significa ripristinarne la continuitá e funzionalitá. L'ingegneria dei tessuti é una combinazione di tutti gli elementi utilizzati in medicina rigenerativa: cellule, scaffold e segnali; il suo scopo é quello di generare tessuti in vitro utilizzando le cellule del paziente, questi poi saranno utilizzati come sostituti per tessuti danneggiati o malati. La principale limitazione dell'ingegneria dei tessuti é la vascolarizzazione, perché le cellule necessitano di essere rifornite con ossigeno e nutrimenti per poter svolgere le loro attivitá fisiologiche ma é anche necessario rimuovere i loro prodotti di rifiuto come il diossido di carbonio.

Fino ad ora non é stata ancora trovata un'adeguata soluzione per vascolarizzare propriamente le strutture che supportano le cellule e che sono utilizzate in ingegneria dei tessuti, ma diversi lavori sono stati effettuati seguendo questo obbiettivo; il presente lavoro analizza i piú importanti, focalizzandosi su quelli che utilizzano i metodi di stampa 3D.

Lo scopo del presente lavoro di tesi é di ottenere uno scaffold vascolarizzato in cui le cellule ricevono un'adeguata quantitá di ossigeno e nutrimenti seguendo la considerazione che in natura ogni capillare é situato ad una distanza di $200\mu m$ da un altro (Figura 1); per questo scopo é stata utilizzata una stampante 3D FDM per stampare una rete vascolare idrosolubile.

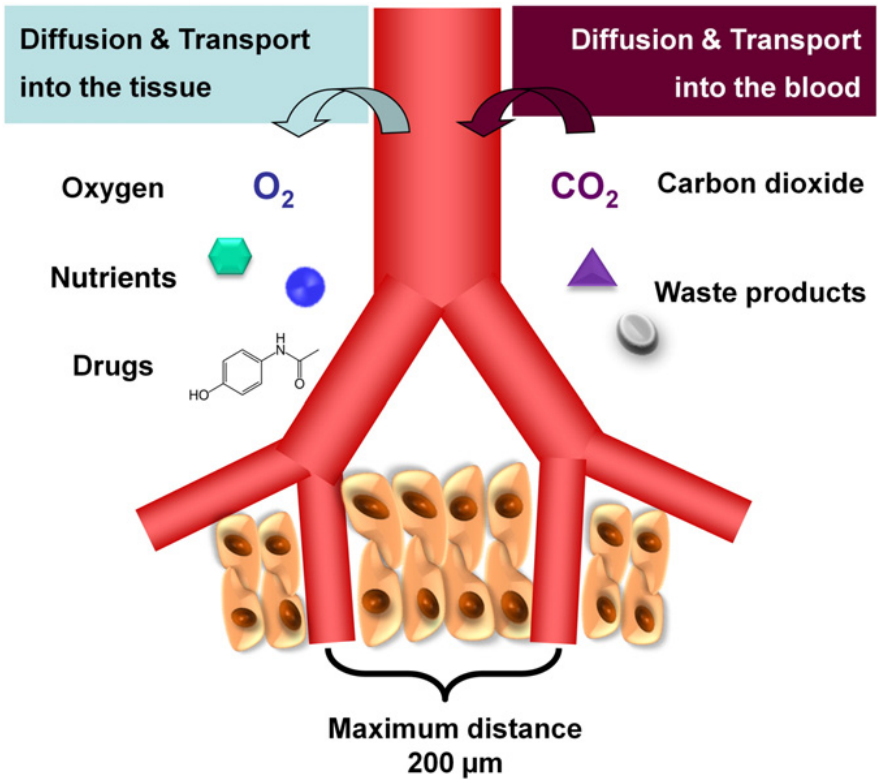
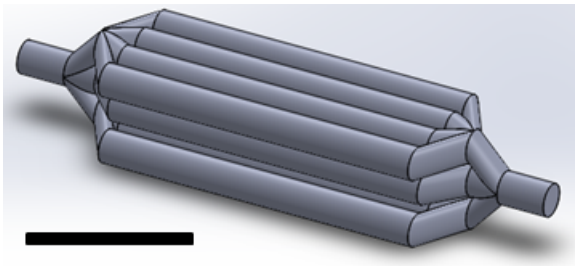


Figure 1: *Descrizione schematica di molecole scambiate tra capillari e tessuti. E' possibile vedere la distanza tra capillari in vivo che é di circa* $200\mu m$.

Materiali e metodi

E' stata progettata una procedura per ottenere un idrogelo vascolarizzato e una rete vascolare avente piani multipli e con canali distanti $200\mu m$ l'uno dall'altro; in questo modo, almeno nella zona centrale, le cellule ricevono un'adeguata quantitá di ossigeno e nutrimenti. Sono stati effettuati calcoli analitici e un'analisi computazionale con il software Ansys Fluent, per definire la velocitá alla quale é possibile perfondere la rete vascolare progettata. La procedura, per ottenere la rete vascolare progettata, richiede una stampante 3D con due estrusori, ma la stampante 3D disponibile in LaBS (Laboratory of Biological Structures) ha un solo estrusore e quindi, é stata stampata una rete vascolare con un unico piano della rete progettata inizialmente (Figura 2).



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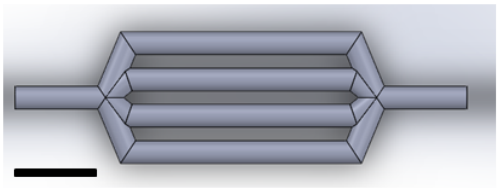


(b)

Figure 2: *(a) Rete vascolare progettata (barra di misura: 10 mm). (b) Rete vascolare stampata con la stampante 3D presente in LaBS (barra di misura: 8 mm).*

La rete vascolare é stampata in alcool polivinilico (PVA), che é un materiale idrosolubile, poi viene inserita in uno stampo per scaffold fatto di gomma siliconica (Silplay 184/28) e inglobata in una soluzione di idrogelo (composta da: 50% di gelatina e 50% di alginato) e una volta che quest'ultima passa dallo stato di sol a quello di gel, la rimozione della rete vascolare viene fatta attraverso la perfusione attiva con acqua distillata.

Lo stampo per scaffold in silicone é stato prodotto da un master ottenuto con la combinazione di due pezzi stampati in acrilonitrile butadiene stirene (ABS) attraverso la stampante 3D FDM.

Sono stati anche progettati un coperchio e una base per sigillare lo stampo per scaffold in silicone per prevenire la contaminazione batterica per il suo possibile utilizzo con idrogeli carichi di cellule, queste parti sono state stampate in acido polilattico (PLA) e processate a posteriori per formare dei fori passanti e filettati in modo da permettere di sigillarle, assemblando il dispositivo finale attraverso l'avvitamento di quattro viti.

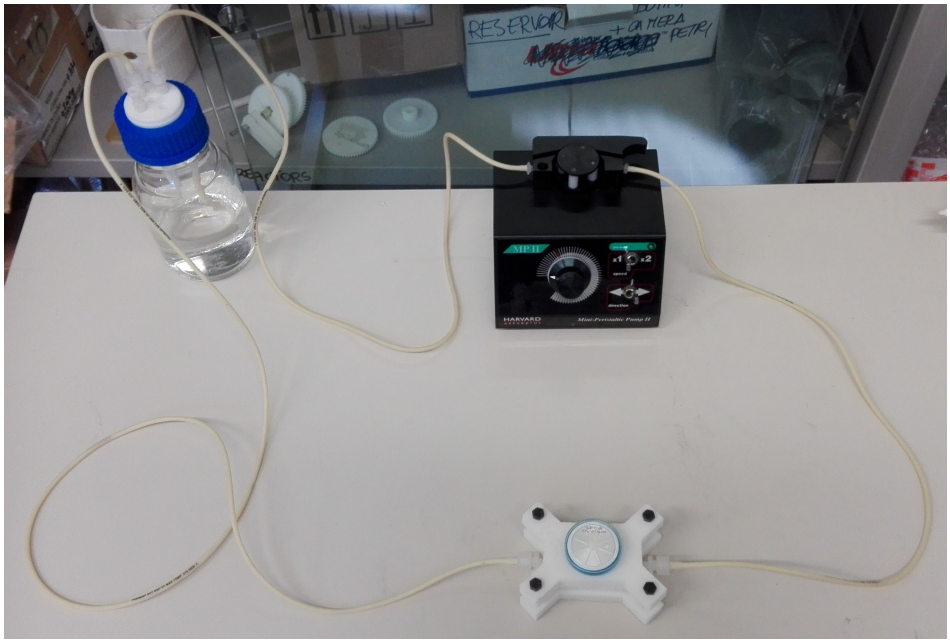


Figure 3: *Nella figura é possibile vedere il circuito idraulico, connesso al dispositivo, il quale é utilizzato per rimuovere la rete vascolare in PVA dall'idrogelo.*

Risultati e discussione

Il dispositivo finale é stato connesso ad un circuito idraulico per rimuovere la rete vascolare in PVA attraverso la perfusione attiva con acqua distillata (Figura 3)
I tempi richiesti per la completa rimozione del PVA sono troppo lunghi (piú di 7 ore), questo puó compromettere la vitalitá cellulare qundo si ha a che fare con idrogeli carichi di cellule, ma é possibile cambiare il tipo di idrogelo o il suo metodo di reticolazione in modo da ridurre questi tempi. Inoltre il diametro dei canali ottenuti nell'idrogelo é nell'ordine di quello di piccole arterie e non di capillari, ma possono essere ridotti utilizzando una stampante 3D con prestazioni migliori e riducendo il tempo richiesto per la completa rimozione della rete vascolare dall'idrogelo. Inoltre i diametri dei canali nello scaffold vascolarizzato ottenuto sono piú grandi di quelli della rete vascolare stampata, questo é dovuto al fatto che il PVA assorbe acqua quando é inglobato nell'idrogelo e durante la perfusione attiva. Quindi é necessario considerare questo fenomeno durante la

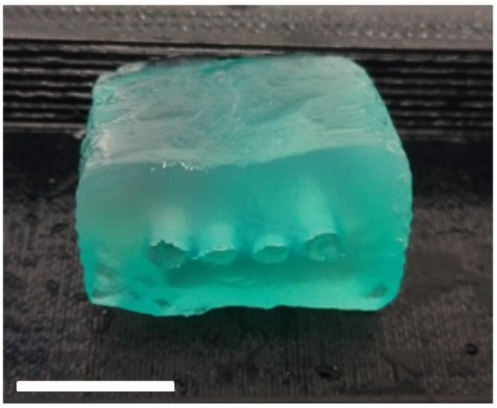


Figure 4: *Nella figura é possibile vedere i canali ottenuti nella zona centrale della sezione laterale dello scaffold vascolarizzato ottenuto con la procedura progettata. Il colore blu é dovuto al colorante per acqua precedentemente utilizzato per colorare la rete vascolare ottenuta nell'idrogelo in modo da poterla vedere (barra di misura: 10 mm).*

progettazione di successive reti vascolari da utilizzare per vascolarizzare scaffold seguendo questa procedura

In Figura 4 é possibile vedere la zona centrale della sezione laterale dello scaffold vascolarizzato, ottenuto seguendo la procedura progettata.

Conclusioni e sviluppi futuri

Il presente lavoro di tesi rappresenta un passo in avanti nella vascolarizzazione di scaffold perhé conferma la possibilitá di utilizzare una procedura economica, recentemente sviluppata per vascolarizzare scaffold.

Negli studi successivi sará necessario provare ad ottenere la rete vascolare su piú piani nello stesso o in un migliore idrogelo per confermare la versatilitá della procedura, la quale puó essere applicata a diversi tipi di reti vascolari e, una volta che é stata ottenuta, effettuare la stessa procedura con le cellule per raccogliere dati sulla reale possibilitá di ottenere costrutti ingegnerizzati vascolarizzati attraverso questa procedura.

Abstract

Introduction

The regenerative medicine goal is to be an alternative to transplantations and prosthesis in order to regenerate damaged or diseased tissues which means to recover their continuity and functionality. Tissue engineering is a combination of all the elements utilized in regenerative medicine: cells, scaffold and signals; its aim is to generate tissues in vitro utilizing cells of the patient, which then will be used as substitutes for damaged or diseased tissues. The main limitation of tissue engineering is the vascularization because cells need to be provided with oxygen and nutrients to perform their physiological activities but also it is necessary to remove their wastes such as carbon dioxide.

Until now has not been found an adequate solution to vascularize properly the structures which support cells in 3D cultures that are utilized in tissue engineering, but several works were performed following this target; the present work analyzes the most important of them, focusing on the ones which utilize the 3D printing methods.

The aim of the present thesis work is to obtain a vascularized scaffold in which cells receive an adequate amount of oxygen and nutrients following the consideration that in nature each capillary is situated at a distance of approximately $200\mu m$ from another one (Figure 5); for this purpose it was utilized a 3D FDM printer to print a hydro-soluble vascular network.

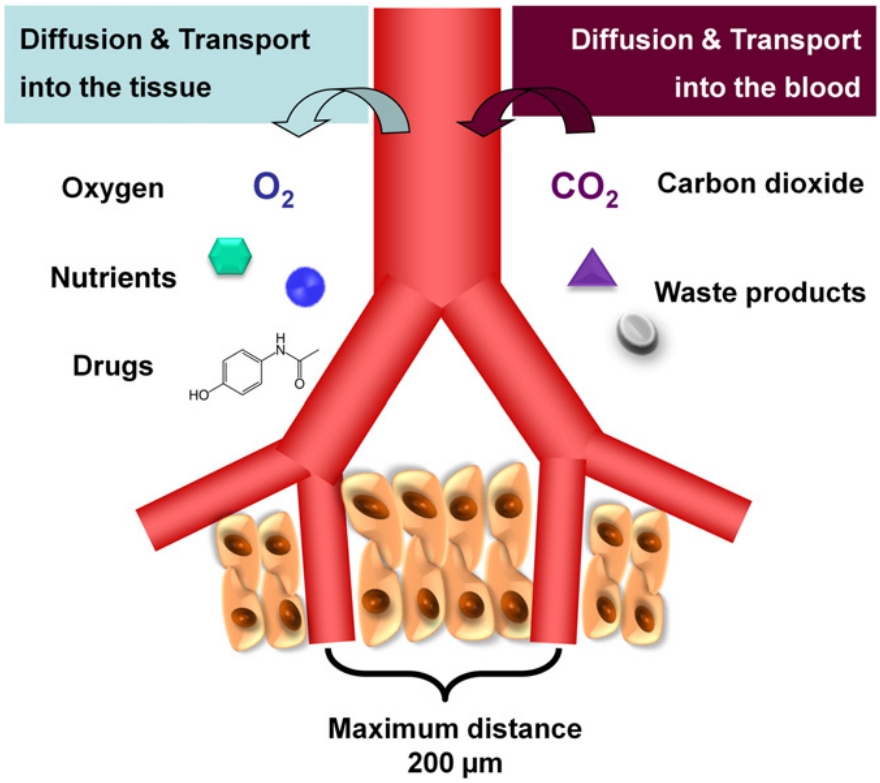
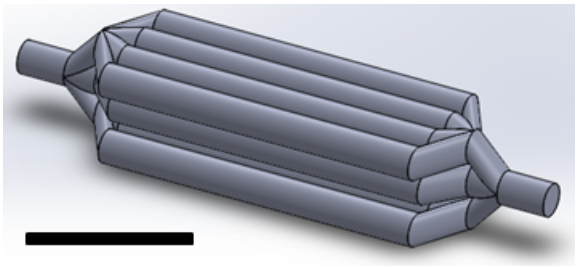


Figure 5: *Schematic description of exchanged molecules between capillaries and tissues. It is possible to see the distance between capillaries in vivo which is about* $200\mu m$.

Materials and methods

It was designed a procedure to obtain a vascularized hydrogel and a vascular network on multiple planes with channels distant $200\mu m$ from one to another; in this way, at least in the central zone, cells receive an adequate amount of oxygen and nutrients. Analytic calculations and a computational analysis through the software Ansys Fluent, were performed to define the velocity at which it is possible to perfuse the vascular network designed. The procedure, to obtain the vascular network designed, requests a 3D printer with two extruders, but the one available in LaBS (Laboratory of Biological Structures) posses only one extruder and so, it was printed a vascular network with only one plane of the originally designed one (Figure 6).



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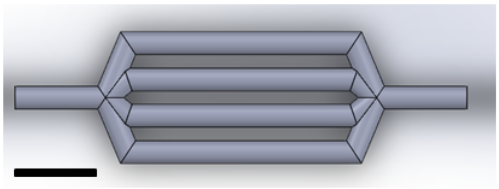


(b)

Figure 6: *(a) Designed vascular network (scale bar: 10 mm). (b) Vascular network printed through the 3D printer present in LaBS (scale bar: 8 mm).*

The vascular network is printed in polyvinyl alcohol (PVA), which is a hydro-soluble material, then it is placed in a scaffold mold made by silicone rubber (Silplay 184/28) and embedded in a hydrogel solution (composed by: 50 % of gelatin and 50% of alginate) and once this last one pass from sol to gel state, the vascular network removal is performed through active perfusion with deionized water.

The silicone scaffold mold was produced from a master obtained with the combination of two pieces printed in acrylonitrile-butadiene-styrene (ABS) through a 3D FDM printer.

It were also designed a cap and a base to seal the silicone scaffold mold in order to prevent bacterial contamination for its possible future utilize with cell-laden hydrogels, these parts were printed in polylactic acid (PLA) and post-processed to form through holes and threaded holes in order to allow to seal them, assembling the final device through the tightening of four screws.

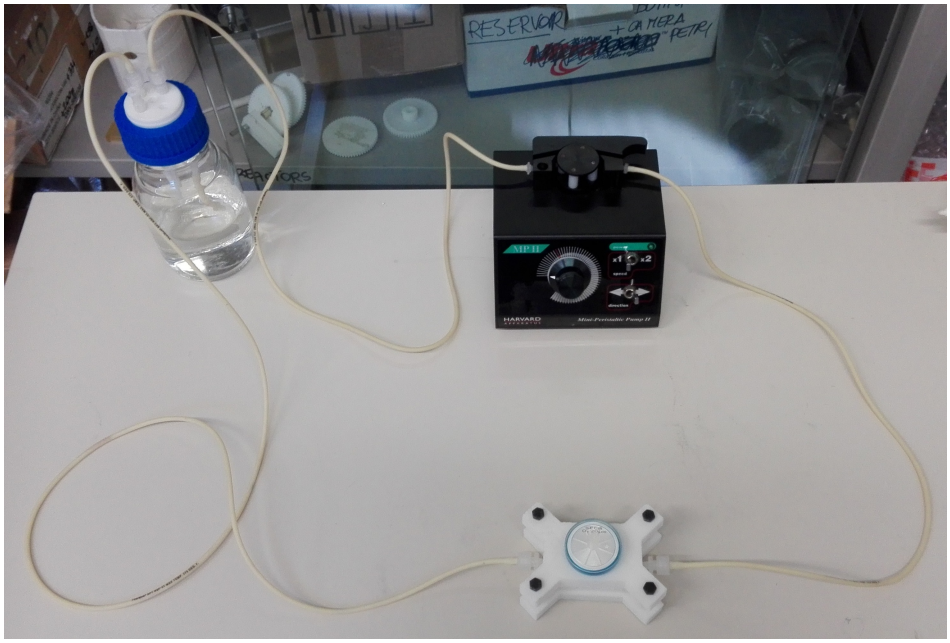


Figure 7: *In the picture it is possible to see the hydraulic circuit, connected to the device, which is utilized to remove the PVA vascular network from the hydrogel.*

Results and discussion

The final device was connected to an hydraulic circuit to remove the PVA vascular network through the active perfusion with deionized water (Figure 7).

The times request for the complete removal of PVA were too long (more than 7 hours), this can compromise cells viability when dealing with cell-laden hydrogels, but it is possible to change the kind of hydrogel or its reticulation method in order to reduce them. Furthermore the channels diameter obtained into the hydrogel is in the range of small arteries and not in the one of capillaries, but they can be reduced using a 3D printer with better performances and reducing the time requested for vascular network complete removal from the hydrogel. Furthermore the channels diameters in the obtained vascularized scaffold were bigger than the ones of vascular network printed, this is due to the fact that PVA absorb water when it is embedded into the hydrogel and during the active perfusion. Thus it is necessary to consider this phenomenon during the design of successive vascular

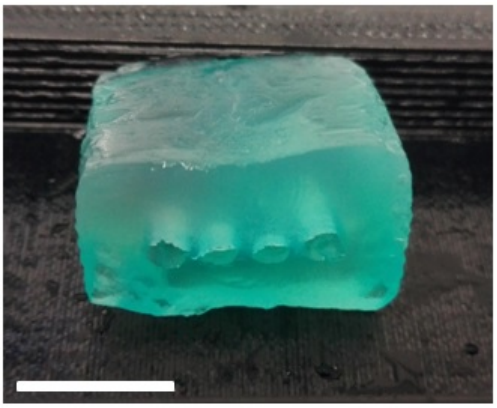


Figure 8: *In the picture it is possible to see the channels obtained into the central zone lateral section of the vascularized scaffold obtained with the designed procedure. The blue color is due to the blue water dye previously utilized to color the vascular network obtained into the hydrogel in order to see it (scale bar: 10 mm).*

networks to utilize in order to vascularize scaffolds following this procedure.

In the Figure 8 it is possible to see the central zone lateral section of the vascularized scaffold, obtained following the designed procedure.

Conclusions and future developments

The present thesis work represent a forward step in the scaffold vascularization because it confirms the possibility to use an economic procedure, recently developed to vascularize scaffolds.

In the successive studies it must be necessary try to obtain the vascular network on multiple planes into the same or a better hydrogel to confirm the versatility of the procedure, which can be applied to different kind of vascular networks and, once it is obtained, perform the same procedure with cells to collect data on the real possibility to obtain vascularized engineered constructs through this procedure.

Chapter 1

Introduction

1.1 Targets

The objective of the present thesis work is to obtain a pre-vascularized scaffold in order to overtake the major limit in tissue engineering which is the lack of vasculature in these structures which are utilized in 3D cell cultures. This is performed through the design and fabrication of a sacrificial structure which is embedded in a hydrogel and then removed in order to obtain a vascular network into the hydrogel. The present work try to produce a scaffold in which the cells present in the core receive an adequate amount of oxygen and nutrients taking inspiration from the fact that in nature each capillary is distant $200\mu m$ from another one.
Tissue engineering purpose is to provide tissues and organs to the patient using a small amount of autologous cells which are seeded on a scaffold and cultured in a bioreactor its principal problem is the vascularization which nowadays still prevent to obtain tissues of large dimensions because cells in scaffold core die due to the lack of an adequate amount of oxygen and nutrients. Through the vascularization an adequate amount of oxygen and nutrients can be provided to the cells preventing their death, in this work are presented several methods to obtain the vascularization of a scaffold focusing on 3D printing because it is the most promising method due to the fact that it is possible to obtain complex structures with different dimensions of channels using several kind of materials. Between

the analyzed 3D printing methods the chosen method to produce the designed structure is: "fused deposition modeling" because it allows the utilize of many materials enabling to choose different solvent to dissolve firstly the supports and then the structure generated through the 3D printer and furthermore this method is cheaper than others.

1.2 Regenerative medicine and tissue engineering

In the last years field of transplantation have been improved with better patients and graft survival but this have caused the increment in demand of organs and tissues which continues to exceed the supply. As result of this transplantation crisis a lot of patients die waiting for a transplant. Thus science have tried to find new solutions to transplantations and one of these is the regenerative medicine [1].

When a tissue or an organ is diseased or damaged it repairs itself through natural processes and these lead to the recovery of continuity but not always to the restoration of functionality. The repaired tissue functionality depends on the criticality of damage and on the kind of tissue [1]. When it is necessary to recovery the functionality, are utilized an organ transplantation or prosthesis but both have some problems:

- *Organ/tissue transplantation* - As previously said the number of patients which need a transplantation have been increased in the last years and this exceeds the organ supply but also after a transplantation patients need to be treated with a life longer immunosuppression [2].
- *Prosthesis* - They do not recovery completely all the functionality and do not last forever but after some time they may fail and need to be replaced [3] [4].

The regenerative medicine purpose is to be an alternative to transplantation and prosthesis in order to regenerate the damaged or diseased tissues which means recovery in continuity and functionality of tissues or organs. Such regeneration can be achieved through cell therapy or tissue engineering [3]. Tissue engineering principal goal is to create new autologous tissues, obtained through the expansions of cells taken from a patient biopsy. In this way are created tissues or organs immunologically compatible which allow to prevent the utilize of immune-depressor drugs [5] [2].
The fundamental components of regenerative medicine are:

- *Cells.*
- *Scaffolds* - They support cell adhesion and growth.
- *Signals* - They induce cells proliferation and differentiation. They can be chemical (for example soluble growth factors) or physical (mechanical, electrical, ...).

These components can be used in any combination or alone, if all of them are used in a therapeutic strategy this one is called "Tissue engineering" and they are combined through the utilize of a bioreactor (Figure 1.1) [1].

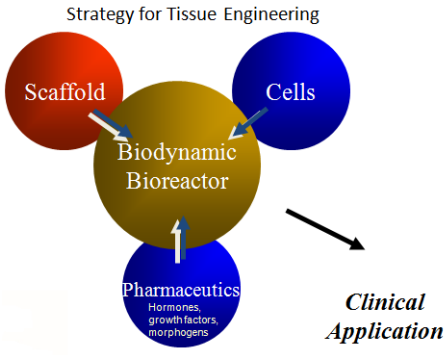


Figure 1.1: *Tissue engineering general scheme. Cells, scaffolds and signals (Pharmaceutics) are combined through the utilize of a bioreactor to produce a mature tissue outside the human body and then utilize it in clinical applications [6].*

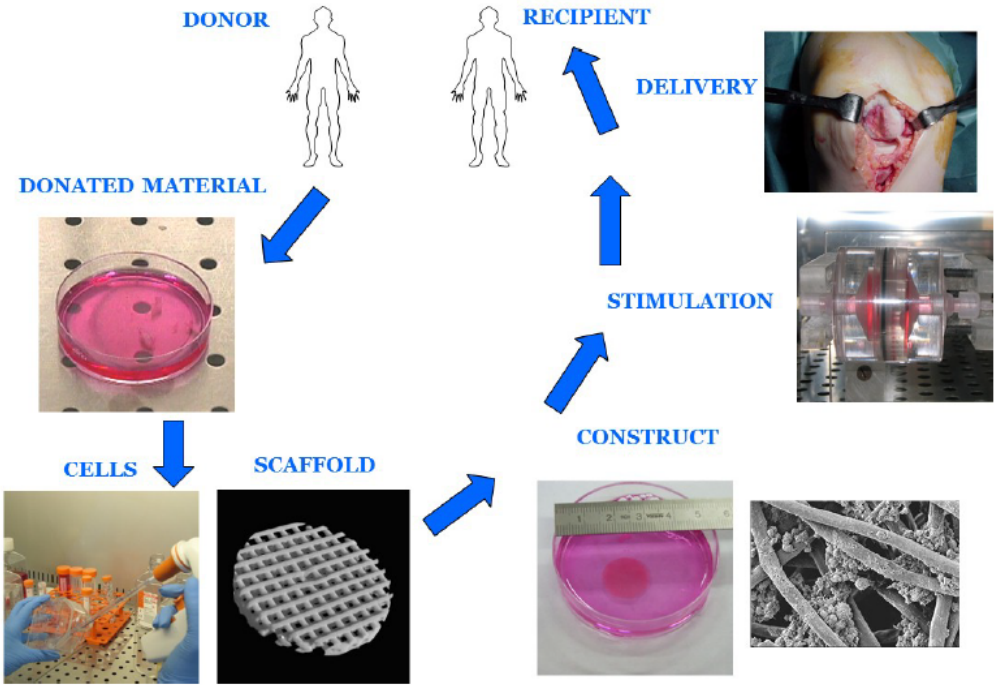


Figure 1.2: *Generic process diagram in a regenerative therapy [1]*

It is possible to say that: in Tissue Engineering we build a part of us outside ourselves, in order to do this, it is necessary to consider that a tissue is made by: cells, extra cellular matrix (ECM) and vascularization. Thus it is necessary to reproduce this organization in order to obtain a functional tissue which can replace the diseased or damaged one present in the patient. Tissue organization is reproduced through the 3D culture of cells onto a scaffold (it simulates the ECM) which needs to be porous and vascularized or allowing vascularization in order to let culture medium in vitro and blood in vivo to reach inner cells, in this way: all the cells are supported with oxygen and nutrients, the metabolic waste are eliminated and chemical signals can reach the inner cells [6] [7].

A generic regenerative therapy process which utilize Tissue Engineering is shown in the Figure 1.2; it consist in: withdraw of cells from the patient, cell expansion, cell seeding on a scaffold, cultivation of the engineered construct through a bioreactor and finally its implantation in the patient.

1.2.1 Cells utilized in tissue engineering

In tissue engineering the cells utilized can be classified based on their immunogenicity (property to provoke an immune response in the recipient):

- *Autologous* - They are patient's own cells or iPS (induced-pluripotency stem cells), these cells are not immunogenic.
- *Syngeneic* - They are cells isolated from tissues donated by patient's identical twin or obtained through the sacrifice of an embryo cloned by patient's somatic cells, these cells are not immunogenic.
- *Allogeneic* - They are cells obtained from other human sources, the immune response caused by these cells can be controlled through immune-depressor drugs.
- *Xenogeneic* - They are cells isolated from other species, these cells cause an acute immune rejection.

Stem cells are able to self renewal without getting old and to differentiate in specialized cells, for these reasons they are good candidates in tissue engineering and more generally in regenerative medicine. The "potency" of stem cells indicates their capacity to differentiate in: all cell families (totipotent cells), multiple cell families (multipotent cells), closely related family of cells (pluripotent cells) or single type of cells (unipotent cells)[6] [1].
The stem cells which can be utilized in tissue engineering are: *embryonic stem cells (ES)*, *induced-pluripotency stem cells (iPS)* or *adult stem cells*.
Embryonic stem cells are isolated from the inner blastocyst which can be obtained through cloning procedure, this leads towards "human therapeutic cloning" which consist in the conditioning of embryonic stem cells to differentiate them in specialized cells in order to obtain specific cells or tissue to repair a damage.
Induced-pluripotency stem cells are obtained from adult somatic cells through genetic modification [8]. These cells have the advantage to overcome the ethical

limitations present in the utilize of embryonic stem cells due to the sacrificial of an embryo.

Adult stem cells are present in all human tissues, they reside in stem cell niches and are responsible of normal tissue renewal (homeostasis) and repair after damage or disease. There are tissue with high adult stem cells fraction (bone marrow, epithelial tissue, vascular endothelium, ...) or low (heart, kidney, cartilage, ...) [1].

In tissue engineering the major potentiality is referable to adult autologous cells in fact their individuation and extraction are well defined furthermore they solve completely the problem of biocompatibility and differentiation but there are problems referred to their expansion in vitro to reach clinically relevant quantities [2]. The proliferative capacity of adult cells derived from some tissue such as: liver, pancreas and nerves is very low [1].

1.2.2 Biomaterials and Scaffolds for tissue engineering

Scaffolds are supports for 3D cells cultures, they allow 3D cell culture until cells produce ECM which then will support them instead of scaffold. In order to do this they must be made by biodegradable material which means that are able to be degraded through biological agents (such as enzymes). Furthermore these biodegradable materials must posses the following characteristics: not to evoke en excessive inflammatory response or toxic effects after implantation in human body, acceptable shelf life, degradation products non toxic and capable to be metabolized by human body, degradation time must balance the process of tissue regeneration, adequate mechanical properties to the tissue of application, easily processable and sterilizable. Scaffolds do not act only as a support for cells but they provide also: *contact guidance* which influence cell adhesion, migration, proliferation, differentiation and *biological, mechanical signals* to stimulate proliferation, differentiation [3] [9].

The biodegradable materials utilized in tissue engineering to produce a scaffold

can be synthetic or natural. *Synthetic biomaterials* (polylactic acid, polyglycolic acid, polycaprolactone, ...) have the advantage to be: versatile (for example they can be produced with different mechanical properties), reproducible and easily processable whereas their disadvantages can be related to biocompatibility problems and the necessity to functionalize them in order to allow cell adhesion. *Natural biomaterials* (collagen, gelatin, alginate, hyaluronic acid, chitosan, ...) have the following advantages: similarity to the natural ECM, high hydrophilicity, degradation products which can be well tolerated by human body whereas their disadvantages are: different properties and problem of immunogenicity depending on their source [3] [5] [9]. It is also possible to utilize *Decellularized scaffolds* to obtain a structure which is identical to the natural tissue in both architecture and mechanical properties but do not contain cells or nuclear material residuals of the organism from which the tissue was withdrawn and then decellularized following decellularization protocols, thus these kind of scaffolds are not immunogenic. Nowadays are studied some bioreactors which are designed to decellularize or recellularize tissues or organs [6].

The structure of a scaffold must be highly porous with interconnected pores to allow: a good cellular penetration in the scaffold core, transport of nutrient also to the inner part of the structure and angiogenesis processes but there is also the necessity to maintain adequate mechanical characteristics which can be reduced due to high porosity. The quantity, interconnection and dimension of pores can be controlled during the production process of the scaffold depending on technique and material chosen. Hydrogels are porous structures which can be used as scaffolds, they simulate an ambient similar to the one at which cells are present in biological tissues because they contain a lot of water thus allow a good solutes diffusion and cellular remodeling. They can be obtained through the reticulation of polymeric materials biological, synthetic or hybrid.[3].

The techniques initially utilized to fabricate porous scaffolds allow to obtain a

casual porosity utilizing physical or chemical methods for pore generation and they comprise: *solvent casting/particulate leaching* and *electro spinning*. In *solvent casting/particulate leaching* procedure the scaffold obtained is a polymeric foam, it is produced through the vaporization of a solvent in which is dissolved a polymer and is present a porous agent which then is removed from the polymer obtained allowing the formation of pores in the structure. The main problems of this technique are: pores not well interconnected and difficulties to control scaffold thickness.

Electro spinning is a technique that allow the fabrication of a scaffold with micro or nano fibers which can be produced with casual or directional orientation, this last one allow to introduce *contact guidance*. The possibility to obtain nano fibers allow a better cell adhesion because cells simultaneously adhere to more than one fiber and this increase the capacity of tissue regeneration. The main disadvantages of this technique are: possibility to utilize toxic solvent, difficulties to reproduce porosity and interconnections of pores [5].

Generally the necessity to have 3D scaffolds with totally interconnected pores, without the utilize of toxic solvent during their production which then need to be completely removed, a good reproducibility and thickness, lead towards the utilize of computer aided techniques which are called 3D printing [3] [10].

An example of scaffold with completely interconnected pores obtained with a 3D printer is the one produced by Barbara Leukers et al. [11]. In this case it was utilized a 3D printer which print a ligand to bind layers of hydroxyapatite powder, the result obtained is shown in the Figure 1.3. In the present thesis work a 3D printer is utilized to fabricate a vascular network and not a porous scaffold; whereas this last one is made by an hydrogel.

Porous scaffolds present some problems in the clinical translations of engineered constructs, in fact the process of endothelialization is difficult for highly tortuous interconnected pores, furthermore they do not have defined inlets or outlets so

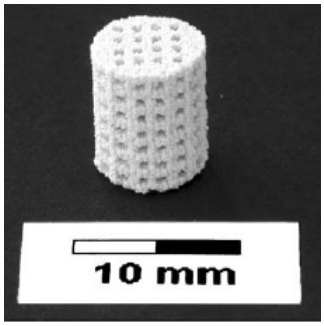


Figure 1.3: *Hydroxyapatite scaffold for bone tissue engineering obtained through the utilize of a 3D printer which print a ligand that is able to bind hydroxyapatite powder. This scaffold have completely interconnected pores, it is reproducible and the 3D printer utilized to print it can be implemented to reach higher dimension if it is necessary [11].*

they cannot be connected directly with the patient's circulatory system. These limitations have lead towards the utilize of scaffolds with defined channel networks in tissue engineering for clinical applications [7].

1.2.3 Bioreactors for tissue engineering

A bioreactor is a device for in vitro development of an engineered construct which is a cell-seeded scaffold. It allow biological and/or biochemical processes in monitored (sensors), controlled (feedback) and automatized environmental conditions. The final purpose of a bioreactor is to develop an engineered construct in as much as possible automatic way which lead to less actions by the operator with lower possibility to contaminate the culture and high reproducibility. Bioreactors are generally utilized for in vitro culture of engineered constructs but they can also be utilized to seed cells on scaffolds or to decellularize organs through chemical protocols [6] [5].

Bioreactors utilized in tissue engineering must monitor some cell culture key parameters (pH, pO_2, pCO_2, temperature) and control them keeping their variations in a physiological range through feedback circuits, furthermore they can provide: fluidic, mechanical or electrical conditioning to develop a tissue with structure and function as much as possible similar to the natural one.

An adequate stimulation of engineered construct provided by a bioreactor allow cells to produce ECM in a shorter time period and in more homogeneous manner than a static culture, it can also induce cell differentiation into a specific phenotype when are utilized stem cells and furthermore cell viability is enhanced due to the dynamic transport of medium which allow the core of a porous scaffold to obtain an adequate amount of nutrients and to remove waste which is difficult to perform in a static culture [12].
In the Figure 1.4 is possible to see a bioreactor utilized by M. Adelaide Asnaghi et al. [13] to seed cells onto a decellularized trachea and culture them to develop a mature tissue for a clinical application.

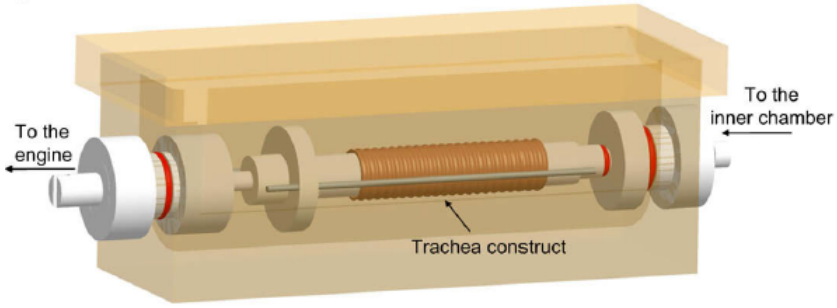


Figure 1.4: *Three dimensional view of a bioreactor utilized for recellularization of a decellularized trachea and culture of the engineered construct obtained for clinical application [13].*

General design specifics for a bioreactor utilized in tissue engineering can be prescriptive or desirable depending on the application and they are:

- *Cytocompatible materials* - These kind of materials need to be utilized for the parts that enter in contact at least one time with cells or culture medium.
- *Sterility* - It is necessary that the materials which enter in contact with the environment in which are present the cultured cells or with the culture medium are sterilizable and that the sterility during cell culture is kept for example in some critical passages like: scaffold fixation, culture medium exchange and cell seeding.

- *Increasing the nutrients and gas transport from culture medium to engineered construct* - It is possible through the movement of culture medium.
- *Physical stimulation of engineered construct during their development* - This is the reason for which have been produced a lot of bioreactors. It allow to obtain a more homogeneous ECM in shorter time period and to differentiate stem cells.
- *Compatibility with laboratory procedures* - It means for example that must be easy to mount under a laminar flow hood [6] [12].
- *Reliability* - The bioreactor must work also without a continuous control by the operator. This is particularly important for the clinical application and less for the research because in this field it is possible to have the necessity to control constantly some variables.
- *No cross-contamination* - It happens when are cultured more than one scaffold with the same bioreactor, in clinical application it have to be avoided but in research sometimes is seek in order to obtain more rapidly several data.
- *Automation and control* - It is necessary to control some variables which are important for cell cultures and keep them in a physiological range.
- *Versatility* - Low modification are required to adapt the bioreactor to other applications different from the original one.
- *Automatic cell seeding* - Utilize the same bioreactor that allow the development of engineered construct also to seed cells onto the scaffold.
- *Reduction of size* - Minimizing the medium volume and reducing the number of tubes.

- *Stand alone* - It means design a bioreactor that works alone without the control of an operator [6].

1.3 The problem of vascularization

The main problem in tissue engineering is the *vascularization* of engineered constructs [9] [7] [14] [15] [16] [17], another relevant issue is their *innervation*; this last one is fundamental to allow the functionality of some tissues (for example muscular tissue [18]) but the problem of vascularization acquire a greater importance to obtain engineered construct with clinically relevant dimensions.
Erik J. Suuronen et al. [19] demonstrated the importance of innervation in a human cornea engineered construct because it enhances the growth of epithelium and the formation of its protective mucin layer; they demonstrated the importance of innervation in the protective quality and function of an engineered tissue furthermore these results are important to consider for the development of any optimized engineered construct for in vitro study and testing purposes.
Whereas Kaufman et al. [18] demonstrated the utility of a surgical operation orientated to transfer the native femoral nerve to promote innervation of a muscular engineered construct which is implanted into a mice to regenerate a damaged muscle and restore its function.
The present thesis work will focus on the problem of vascularization which nowadays is the main challenge in tissue engineering because cells present in the scaffold core die if the engineered construct have a thickness major than $400\mu m$ due to a non adequate support of oxygen, nutrients and elimination of waste products. Furthermore the vascularization is important also for the influx of circulating cells and bioactive factors into tissue [10] [7]. Finally lymphatic vessels which are another fluidic network are needed to regulate interstitial fluid volumes present in the tissue. Until these fluidic networks can be fabricated engineered construct will not recapitulate the functions of natural tissues [7].

Actually only some tissues like: skin, cartilage, cornea and bladder can be supplied with oxygen and nutrients by blood vessels further away, for this reason they can be reproduced in vitro and have been born some clinical applications using these tissues [20]. Almost all other tissues in human body have a blood vessel network with vessels at an optimal distance smaller than $200\mu m$ which is correlated to the diffusion limit of oxygen (Figure 1.5) [9].

Large porous engineered constructs can be perfused through a bioreactor, in vitro their inner cells are well supplied by oxygen and nutrients but in vivo the oxygen/nutrients diffusion is limited by insufficient distances between the capillaries and the engineered construct core [9].

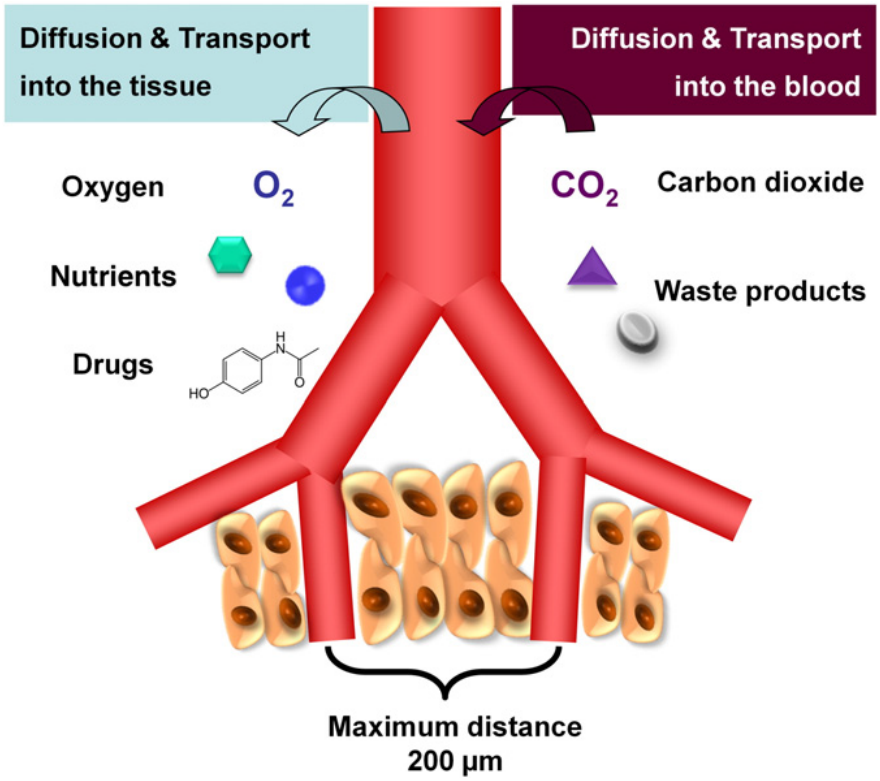


Figure 1.5: *Schematic description of delivered molecules from capillaries to surrounding tissues (nutrients, oxygen and drugs) and from tissues to capillaries (carbon dioxide and waste products) in vivo. It is possible to see the maximum distance between two capillaries which is of* $200\mu m$ *[9].*

1.3.1 Anatomy and physiology of human blood vessels

In order to overcome the problem of engineered construct vascularization in tissue engineering it is necessary to study anatomy and physiology of human blood vessels.

Blood vessels are classified based on their capacity to conduct blood from heart to tissues or vice versa and on their dimensions. Arteries and arterioles conduct blood from heart to capillaries present in tissues whereas venules and veins take blood from capillaries and carry it to heart.

Arterioles, venules and capillaries can be observed only at the microscope and so they constitute the *microcirculation* [21].

All human blood vessels have an inner coating of endothelial cells called *endothelium* which prevents thrombogenesis and is a cellular barrier that must be penetrated to deliver molecules to the tissues or from this ones to the vessel lumen. Gases and smaller molecules can pass through the gap between cells whereas bigger molecules are transported through the endothelium towards their destinations [9].

Capillaries which are the smaller blood vessels are constituted only by a single layer of endothelial cells (*endothelium*) and by a basal lamina; the walls of all other blood vessels contains variable quantities of smooth musculature and fibrotic and/or elastic connective tissue. Fibrotic tissue is composed by collagen which is a protein that allow blood vessels to extent with the application of blood pressure without tearing whereas elastic tissue is composed by elastin which is a protein that let blood vessels to expand or contract when the inner pressure change.

Arteries have big diameter and wall thickness, except aorta they have a diameter comprise between $2mm$ and $6mm$. The bigger arteries provide low resistance to blood flow thus their duty is prevalently to conduct blood, their walls are composed by: smooth musculature, fibrotic and elastic tissues.

Arterioles branch out from arteries and flow into a capillary bed, they have an average diameter of $0.03mm$ and they contain a lot of smooth musculature but low elastic tissue, this is connected to their function of hydraulic resistance regulators, in fact through the muscular contraction their lumen is reduced and this increment the flow resistance.
Capillaries have a small diameter comprise between $0.005mm$ and $0.010mm$; their function is to allow molecules exchanges between blood and cells present in the tissues, thus they are composed only by the endothelium and basal lamina. Almost all cells are situated at less than $1mm$ from a capillary and the capillaries are not isolated but they form networks called capillary beds. Due to capillaries ramifications their total transversal area is bigger than the one of other blood vessels and so the velocity of blood which flow in them is reduced, in fact it is like a channel which increase its original area: the fluid which flows in it have more surface available and so its velocity is reduced. Blood flows in capillaries with a velocity approximately of $0.1\frac{mm}{s}$ [21]. The permeability of capillaries variate in different kind of tissues and so it is possible to distinguish them into:

- *Continuous capillaries* - They are the most common capillaries and their endothelial cells are close to each other. These kind of capillaries limits the passage of big molecules because they cannot cross easily the endothelial cells membrane neither the gap between cells.

- *Fenestrated capillaries* - The endothelial cells which form these kind of capillaries have relatively large pores which allow big molecules to pass easily through them. Thus they are highly permeable to both small an big molecules and so generally are present in organs where it is necessary a fast molecules exchange between blood and cells in the tissues to perform their function.

Venules are found after capillaries, toward heart, they contain low or not smooth musculature and their average diameter is $0.02mm$. Smaller venules are more like

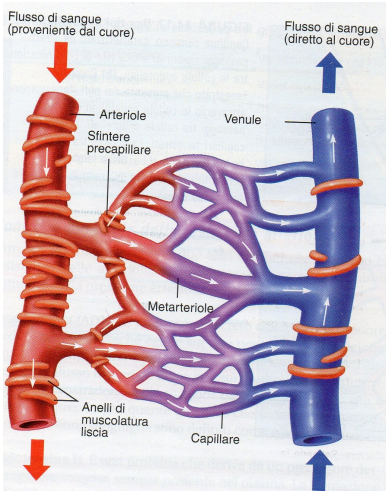


Figure 1.6: *In the figure it is possible to see all the main components of the microcirculation (arterioles, capillaries, metarterioles, venules and precapillary sphincters) and their organization[21].*

capillaries than arterioles, this is due to their lack of musculature layer and so they allow exchanges between blood and interstice. Thus not only capillaries but also venules allow this function.

Veins are present after venules, toward heart, they have a low wall thickness because they need to withstand low blood pressures and an average diameter of $5mm$. The composition of their wall comprise: smooth musculature, fibrotic and elastic tissue; they contain unidirectional valves in the peripheral veins but not in the ones nearest to heart, these valves allow blood to flow only toward heart and so prevent the backflow.

Microcirculation (Figure 1.6) comprise also *metarterioles* which are blood vessels with an intermediate structure between arterioles and capillaries, they have isolated regions of smooth musculature and connect directly the arterioles to venules allowing blood to pass through them also when the contraction of arterioles prevent its flow in capillaries. The flow in capillaries is also controlled by *precapillary sphincters* which are ring of smooth musculature that surround their arteriolar extremity [21].

In the Figure 1.7 is possible to see the dimensions of diameter and the main characteristics of blood vessels.

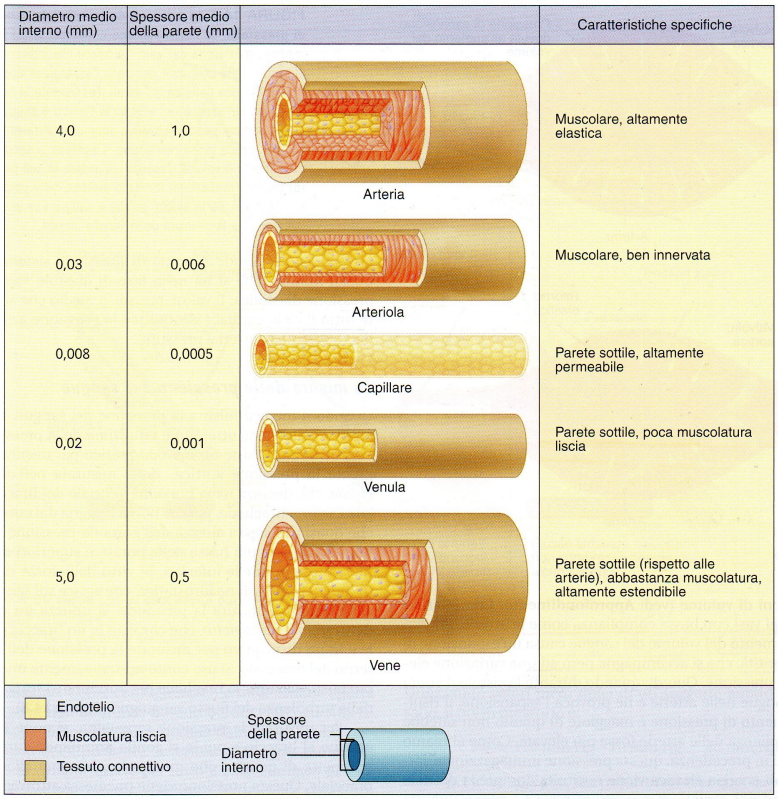


Figure 1.7: *Diameter and principal characteristics of blood vessels [21].*

New blood vessels can be formed through two different methods: *vasculogenesis* and *angiogenesis*.

Vasculogenesis is the production of new blood vessels starting from the aggregation of endothelial progenitor cells and hematopoietic stem cells in blood islands, then the endothelial progenitor cells differentiate in endothelial cells which gradually fuse and form primitive vessel networks within the ECM [15]. Subsequently a process of angiogenesis lead to the creation of a more complex vascular network. Vasculogenesis is not only a process which occurs during embryonic development but it happens also in the adult life [9].

Angiogenesis can occur via sprouting or splitting routes. Sprouting consist in the

expansion of vascular network from the pre-existing capillaries and is guided by tip cells, whereas splitting involves the internal division of an existing capillary into multiple capillaries. In sprouting the capillaries can migrate towards pre-existing vessels and integrate with them [15]. Angiogenesis is a process which request an high regulation provided by the interaction between endothelial cells and non endothelial cells. Furthermore a chronologically precise adjustment of vessel growth, vessel maturation and finally suppression of endothelial cells is required for the formation of physiologically functional vessels [9].

1.4 State of Art: Different approaches for scaffold vascularization

There are two main strategies utilized to obtain vascularized engineered construct: the first one is *cell-based strategy*, it utilize the intrinsic ability of endothelial cells to form new vessels which is known as vasculogenesis or angiogenesis whereas the other one is *scaffold-based strategy*, it consist in build vascular network directly in the scaffold or utilize decellularized tissues which maintain their native vascular networks [9].
In the case of *cell-based strategy* it is possible to obtain prevascularized engineered construct through two ways:

- *In vitro prevascularization* - Endothelial cells are seeded on a scaffold and are cultured in vitro also with other kind of cells, in this way they tends to aggregate forming blood vessels, then the construct obtained is implanted in vivo.

- *In vivo prevascularization* - The scaffold is implanted in vivo in a non damaged zone to allow its vascularization and then it is removed and implanted in the lesion site. The main disadvantage of this procedure is that it request at least three surgeries.

The aim of scaffold prevascularization is to promote the anastomosis between blood vessels obtained in the engineered construct and the ones present in human tissues after its implantation [9] [22].

A disadvantage of in vitro prevascularization is the difficulty in the spatial control of the vascular network formation and for this reason are utilized biochemical and biophysical cues which enable a greater control of vasculogenesis and angiogenesis processes [15]. The main strategies utilized to control vasculogenesis and angiogenesis consist in the utilize of: *angiogenesis growth factors* which guide these processes generating a chemical gradient, *cytokines* which act as indirect angiogenic growth factors or *proteins* which support adhesion and migration of cells [9]. The utilize of these biochemical cues can also be combined with materials and scaffolds designed to enhance their vascularization [10].

In the case of *scaffold-based strategy* can be utilized: decellularized tissues which maintain their vascular network or porous scaffolds in which, using different methods, is created a network of vessels to provide the same function of the native vascular network.

Decellularized tissues present the advantages to maintain the whole biological structure as it was in native condition and to have a good biocompatibility, but it is difficult to obtain a complete recellularization of the matrix obtained through the decellularization process and furthermore they lack of reproducibility which can be reached through the creation of a vessel network within a scaffold [9].

Several strategies were applied to obtain a vascularized scaffold through the construction of a vascular network within the scaffold, some of them can be considered as *engineer methods*, others are derived from *micro-fabrication techniques*, whereas more recently the *3D printing* technology assumed an increasingly important role due to the capacity to obtain a lot of shapes and so also more complicated vascular network can be printed through these innovative techniques.

An example of engineer method which is simple but important is the *needle mold-*

ing technique (Figure 1.8), it consist in a mold where are inserted needles, in the mold is poured an hydrogel solution and after its gelation the needles are removed, in this way it is possible to obtain channels with a diameter of $75 - 150\mu m$ which approximately is the dimensions of arterioles. This technique is very simple and do not allow to obtain complex vascular networks but it is important because channels with dimensions able to be connected to a pump were created and they were seeded with endothelial cells and then perfused [23] [7] [24].

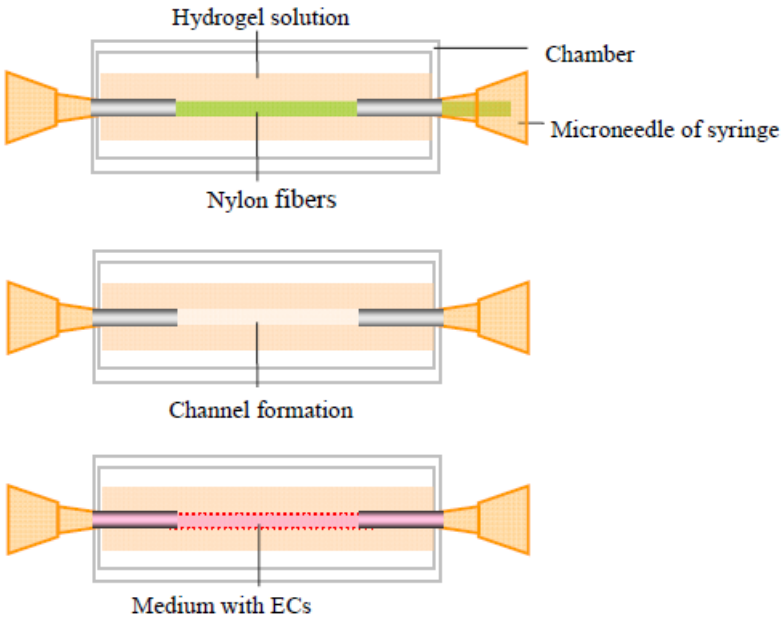


Figure 1.8: *Procedure of a needle molding technique which allow to obtain channels within a hydrogel [24].*

The *modular assembly technique* (Figure 1.9) combine both scaffold-based and cell-based strategies, it consist in the creation of a thick and vascularized tissue through the encapsulation of endothelial and other tissue's cells in small modules made by hydrogel which are packed and perfused with culture medium or blood [23] [24]. Through this technique it was demonstrated that after 7 days of transplantation the modules were connected with host vasculature and were also reported different shape of modules utilized (spheres, cylinders, disks, ...) [23].

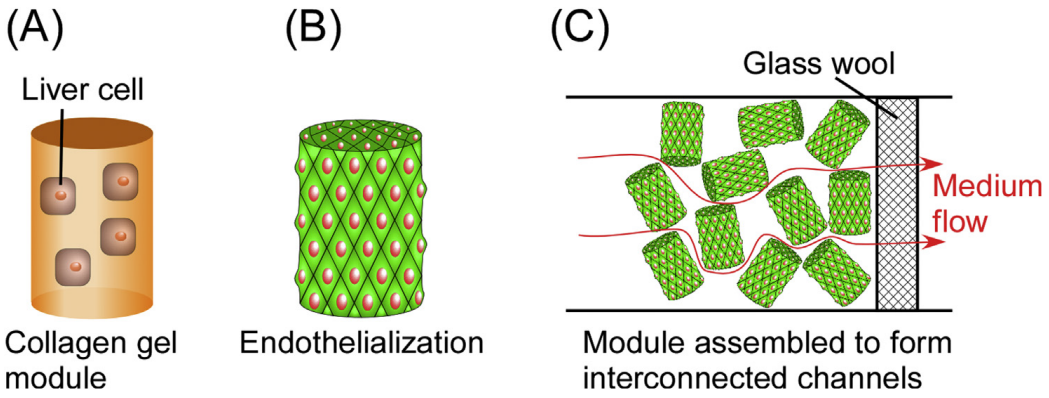


Figure 1.9: *Assembly of hydrogels modules containing cells.(A) Tissue cells (in the figure liver cells) are encapsulated in hydrogel modules.(B) Hydrogel modules are endothelialized.(C) Packing and perfusion of the hydrogel modules [23].*

The *micro-fabrication techniques* consist in the utilize of photo-lithography and soft lithography methods to create a particular designed shape through which is molded an hydrogel containing cells and then this last one is assembled with a flat slab of hydrogel creating the channel network within it (Figure 1.10) [23] [7] [24]. These techniques are very important to obtain vascularized scaffold because they allow the creation of vascular network with dimensions similar to the ones of native vasculature (channels diameters: $50 - 100\mu m$ and distance between channels: $100 - 200\mu m$), but their major limitations are due to: the creation of a complex but single layers of vascular network which can be partially solved by overlapping more vascularized hydrogel layer but it is a difficult process also for the layers alignment and furthermore another issue is that the channels obtained are rectangular so the wall shear stress is not homogeneous, this cause a different stimulation of endothelial cells depending on their position in the channel even if this last drawback is partially reduced by the tendency of endothelial cells to change the shape of the channel from rectangular to cylindrical [23] [7].

Through the *3D printing* techniques it is possible to obtain more complex vascular network because they enable a free form fabrication and furthermore they have a restricted cost; for these reasons the rapid prototyping techniques have

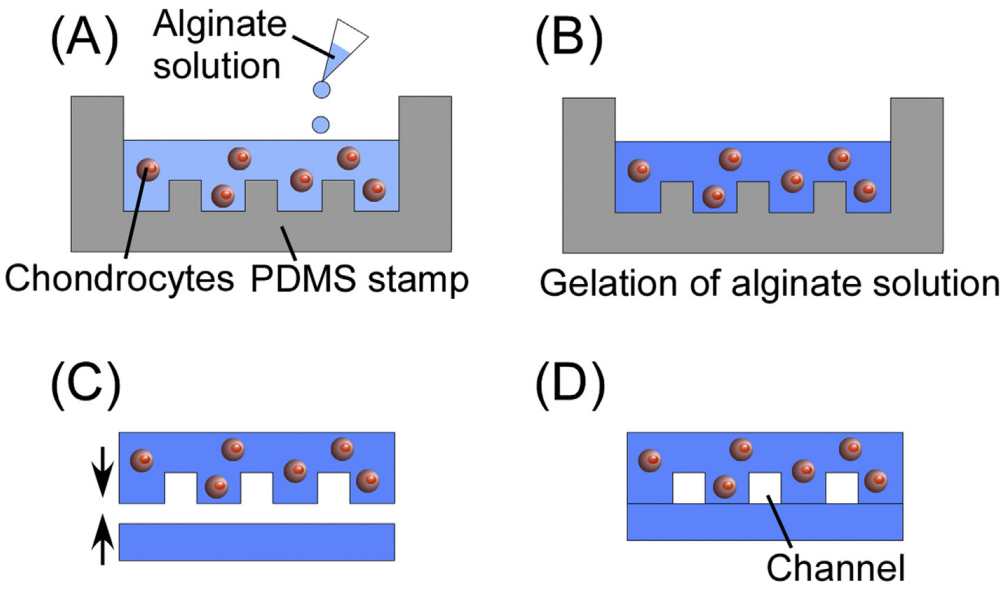


Figure 1.10: *Micro-fabrication technique which utilize the soft lithography to obtain a PDMS stamp through which is obtained the vascularized hydrogel containing cells.(A) The hydrogel solution (alginate in this case) containing cells is poured into the PDMS stamp obtained with the soft lithography. (B) Gelation of the hydrogel. (C,D) Assembling of the molded hydrogel with a slab of hydrogel in order to create the channels [23].*

the potentiality to overcome the needle molding and micro-fabrication techniques utilized for the scaffold vascularization [7]. Even if the 3D printing, which allow to obtain a high resolution or bioprinting, are not too much economic.

1.4.1 3D printing

This group of techniques utilizes different ways to create a designed structure, but all of them build it layer by layer through the utilize of a slicing software which slice the drawing obtained with a computer-aided design (CAD) software and generate a G-code which is used by the 3D printer to obtain the structure. In the G-code are present the information which allow the 3D printer to move the effector in the space and build layer by layer the structure by: depositing material, solidifying resin, fusing powder, ... These techniques follow a *bottom up* approach which means that they start from the little components of the final structure to obtain it and do not start from a bigger part of material to obtain it like in the

top down approach [10] [3] [9].

The 3D printing techniques allow a great control in the space and so enable to obtain complex structures which cannot be obtained through the traditional manufacturing techniques, this allow to fabricate structures more similar to the biological tissues, furthermore due to the use of a CAD drawing these techniques can also interface with medical imaging like magnetic resonance images (MRI) and build scaffolds with complex architectures which can fill properly defects in the patient's tissues and expedite tissue regenerations [10].

Between the several available 3D printing technologies, the ones which can be used for scaffold vascularization, from which some of them were also adapted for the bioprinting are: FDM (Fused Deposition Modeling), SLA (Stereo Lithography Apparatus), DLP (Digital Light Processing) and SLS (Selective Laser Sintering).

1.4.2 FDM (Fused Deposition Modeling)

This technique allow to print thermoplastic polymers by fusing a filament of material (generally PLA, ABS, PVA, ...) which pass through a zone in the extrusion head that warm it until it is fused and then it is extruded by a nozzle whose diameter generally can vary from $0.2mm$ to $0.4mm$ whereas the diameter of the filament which enter in the print head generally is of $1.74mm$ (Figure 1.11). Depending

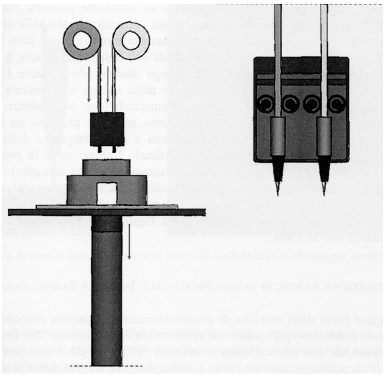


Figure 1.11: *Schematic representation of 3D printer apparatus which work using FDM technology. It is possible to see that the filament is pushed into the extruder by a roller, in this case are represented two extruder and so two rollers: one for each extruder [5].*

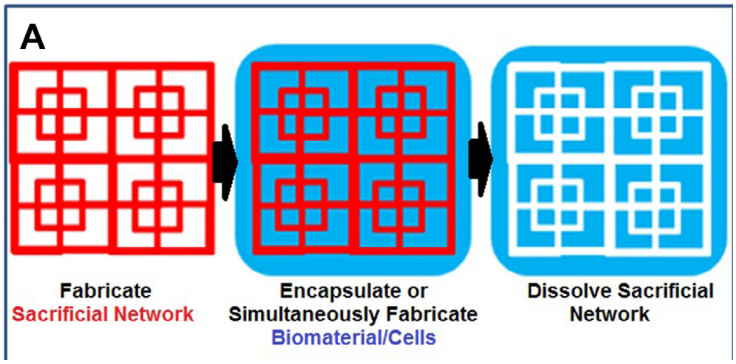
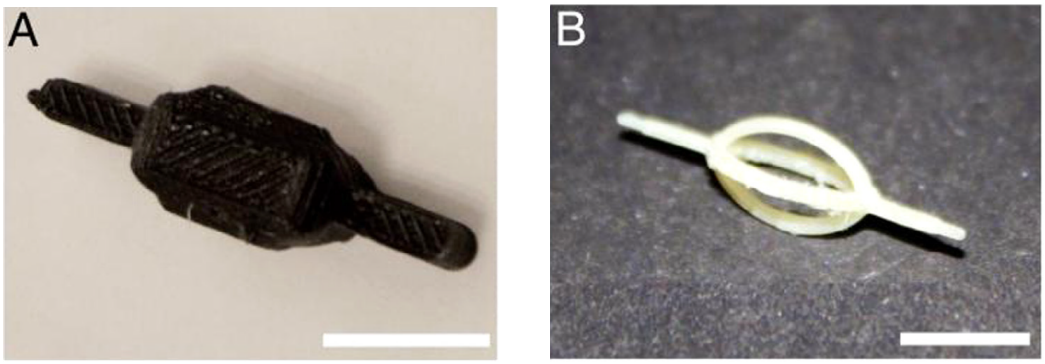


Figure 1.12: *Schematic representation of a scaffold vascularization technique which consist in the production of a sacrificial network which then it is embedded and dissolved in a structure which may contain cells [15].*

on the kind of 3D printer commercially available which use FDM technology the extrusion head and the bed are moved in the space along the x,y and z axes to deposit the extruded filament layer by layer in order to obtain the designed structure[10] [3].

The present thesis work utilize this kind of 3D printing to obtain a water soluble vascular network which is embedded in a hydrogel and then eliminated through water perfusion to obtain a vascular network in it. Thus the present approach consist to obtain a sacrificial template to mold a scaffold which may contain cells (Figure 1.12) and it was used in several works as a way to obtain a vascularized scaffold [15].

The work of Rodrigo Pimentel C. et al. [17] inspired the present thesis work because in it, they utilized a 3D printer commercially available with two extruders which, through the FDM technique, allowed to print a vascular network overcoming one important limitation of the previously utilized methods which might not to be limited in complexity nor in dimensions but were restricted on a single plane development and so they do not represent the complexity of real tissues. The vascular network printed was made by two different materials: PVA (utilized to print the structure) and PLA (utilized for the supports); this is due to the possibility of printing simultaneously two different kind of material thanks to the



(a) *(b)*

Figure 1.13: *The picture captured by Rodrigo Pimentel C. et al. allow to see the printed structure surrounded by the supports (A) and the structure obtained after the supports dissolution (B). In the figure it is possible to see PVA in white color and PLA in black color (scale bars: 6 mm) [17].*

double extruder. In this way it was possible to extend the vascular networks to more than a single plane because the supports allow to print overhanging vessels and they can be removed through a different solvent which do not dissolve the structure (Figure 1.13).

After the supports dissolution, through chloroform, the structure obtained was embedded in a cell-laden hydrogel made by gelatin, which gelation was performed thermally and chemically through the utilize of the protein transglutaminase and finally the structure was dissolved through the perfusion with culture medium once the hydrogel became solid. The authors were not able to obtain channels with a diameter minor than $1mm$ in the final hydrogels even if the diameter designed in their CAD models were about $0.6mm$ and this is due to the fact that during their dissolution the PVA structure, which will form the channels within hydrogel incorporates the medium utilized for its removal incrementing its diameter. However they introduce a method which can be used also with other complex vascular network strengthening the utilize of commercially available 3D printers which use FDM technology for tissue engineering applications. In Figure 1.14 it is possible to see the vascularized hydrogel obtained by the authors.

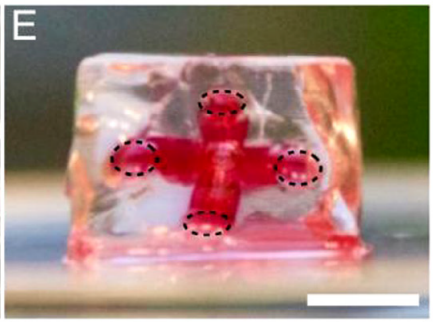


Figure 1.14: *The picture obtained by Rodrigo Pimentel et al. shows the cross section of the vascularized hydrogel central zone (scale bar: 5 mm) [17].*

Some 3D printing using the FDM technology which are commercially available were modified in order to print biological material by several researchers; in this case it is possible to talk about a bioprinting technology even if the ink printed may not contain cells [25]. An important example of a commercially available 3D printer modified to print a carbohydrate glass mixture was the work performed by Miller et al. [26]. In this work the authors modified a 3D printer with a syringe containing a mixture of carbohydrate glass to print a lattice with this material and then put it in a mold where was casted an hydrogel solution containing cells, after the gelation process around the printed lattice, the carbohydrate glass structure is removed by washing it through culture medium and a vascularized hydrogel is obtained (Figure 1.15).

Furthermore the vascular network has been seeded with endothelial cells and perfused with blood under high-pressure pulsatile flow. It was observed that endothelial cells formed sprouts which extended from the patterned vasculature into the bulk gel, this means that the pattern vasculature act as a guide for the endothelial cells, but these ones can also develop angiogenesis processes and form new vessels from the ones engineered to provide the sufficient amount of oxygen and nutrients to all the cells present in the hydrogel. The strong points of this work are the biocompatibility and low cost of carbohydrate glass, however, even if this one has a high capacity to be printed with overhanging parts, due to its mechanical prop-

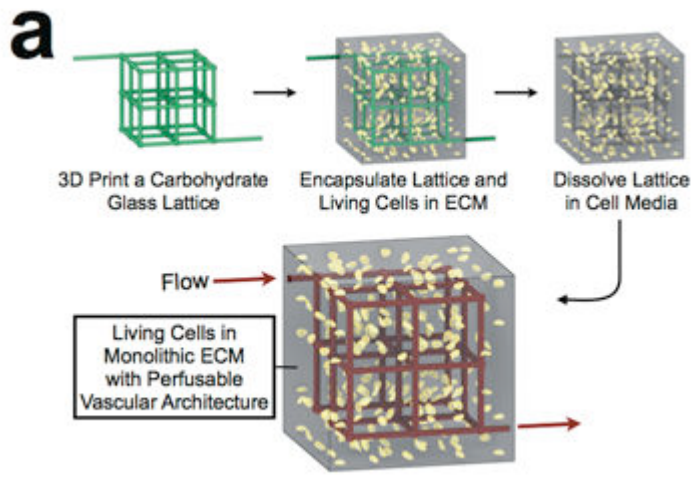


Figure 1.15: *In the figure it is possible to see the procedure utilized by Miller et al. to obtain a vascularized scaffold. A carbohydrate glass lattice is printed through a modified 3D printer and then it is embedded in a cell laden hydrogel, finally the carbohydrate structure is removed leaving a vascular network in the hydrogel [26].*

erties, it do not allow to obtain too complex structures like 3D networks which simulate the native vasculature structure and furthermore this process requests the modification of a commercially available 3D printer in order to print biological material and this implicate a more complex procedure to obtain a vascularized hydrogel.

1.4.3 SLA (Stereo Lithography Apparatus)

The stereo lithography technique has been the precursor of all 3D printing techniques and it is based on the solidification of photo-sensible polymer resins layer by layer through the passage of a UV laser, after one layer is completed the platform on which is being built the structure is lowered in the reservoir containing the polymer resin and so a new resin layer is available for solidification through the laser (Figure 1.16). Once the structure is obtained the liquid resin in excess is washed out and the structure polymerization is completed with a supplemen-

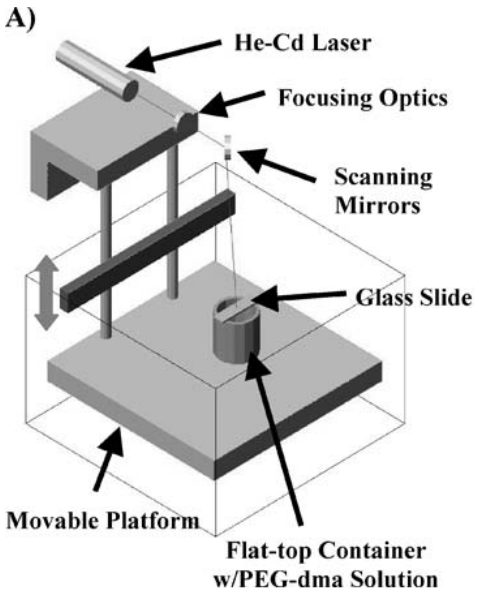


Figure 1.16: *In the figure it is possible to see a generic Stereo Lithography Apparatus which utilize a He-Cd Laser to produce an electromagnetic radiation in UV specter to photo-polymerize the polymer resin present in a container layer by layer in order to obtain the final polymeric structure [27].*

tary exposure to UV light. The resins utilized in SLA are cytotoxic and so the commercially available 3D printers which utilize this technology cannot be used for tissue engineering applications but some researchers modified them in order to print photo cross-linkable hydrogels which may also containing cells [5] [10].
Karina Arcaute et al. [27] have modified a commercially available 3D printer, which utilize SLA technique, to print poly(ethylene glycol) (PEG) hydrogels, which may contain cells, instead of polymeric resins; thus they fill the reservoir which normally contain the resin with a hydrogel solution which is crosslinked through UV light projected by the laser, this last one in commercially available 3D printers which utilize this technique has a spot which can vary from 250 to $75\mu m$ of diameter and so it is possible to obtain also a high resolution. The authors made several tests to understand how to set the parameters like: hydrogel concentration, laser energy and photo initiator concentration in order to obtain a crosslinked hydrogel from its solution through this technique. According to the

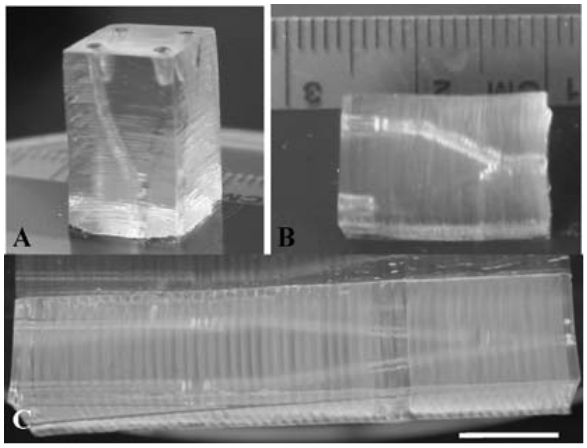


Figure 1.17: *The picture captured by Karina Arcaute et al. shows three blocks of hydrogel with embedded bifurcated channels obtained with their 3D printer which utilizes SLA technique and it has been modified to print PEG hydrogels solutions instead of polymer resins (scale bar: 10 mm) [27].*

authors results, the photo initiator, is cytotoxic only in high concentrations and elevated exposure time. Furthermore they observed that the viability of fibroblasts present in PEG hydrogel solution printed with this technique remains high even one day after the printing process and they realized some structures which have a certain importance for tissue engineering such as a hydrogels in which are present channels of $1mm$ diameter (comparable to the dimensions of a small artery) with bifurcations (Figure 1.17).

1.4.4 DLP (Digital Light Processing)

The 3D printers which utilize DLP (Digital Light Processing) technique project an UV light on a digital screen made by pixel to reflect it with the image which correspond to the current layer and photo polymerize layer by layer a polymer resin present in a reservoir, where the plate on which the structure is built goes down in it, after the solidification of each layer, by a distance which corresponds to the layer thickness to obtain the complete structure (Figure 1.18). This technique is similar to SLA (Stereo Lithography Apparatus) but it utilizes a digital micro mirror instead of a laser and this increment the process speed because no matter how it is

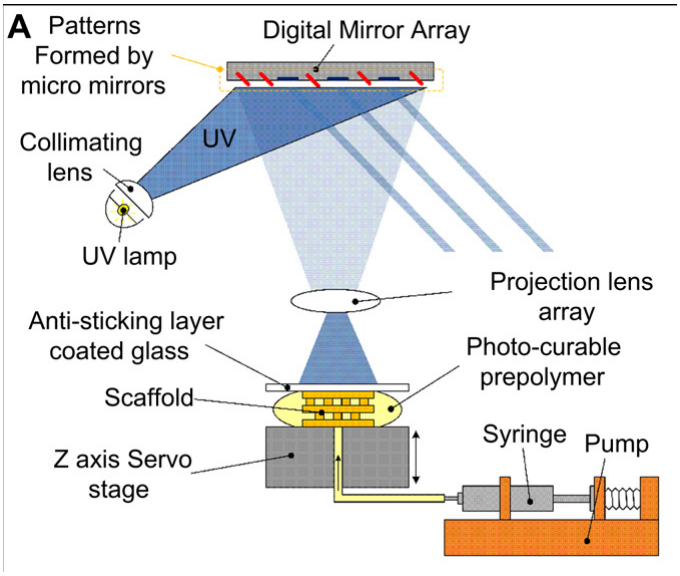


Figure 1.18: *In the figure is shown a schematic representation of a 3D printer which utilize DLP technique to obtain a polymeric structure from a polymer resin. In particular it is possible to see the digital mirror device which reflect the UV light projected on it with the different sections of the designed structure obtained with a slicing software [29].*

complex the layer, it is printed with the same time of the others because each layer is projected entirely [28]. Such as the 3D printers which utilize SLA technique, it is not possible to use 3D DLP printers in tissue engineering application due to the non biocompatibility of the resins but recently some new resins which are more biocompatible have been produced and furthermore some researchers proposed modifications on commercially available 3D printers which utilize DLP technology or on parts of them in order to print biological materials (photo cross-linkable hydrogels) changing this technique in a bioprinting and enabling its utilize for tissue engineering applications [10].

Robert Gauvin et al. [29] have modified a 3D printer which utilize DLP technique to print gelatin methacrylate (GelMA) hydrogels and through it they printed several scaffold which, after the fabrication process, were seeded with immortalized human endothelial cells. GelMA hydrogels were utilized due to the presence of adhesive peptides such as RGD at which integrins can bind, but they need to be

modified with methacrylamide to produce gelatin methacrylate in order to obtain a photo cross-linkable gelatin. The authors proposed some future developments of this technique which will involve the printing of cell-loaded GelMA instead of seeding the structures after their fabrication and so this technique can also be used for printing of vascularized scaffold which contain cells.

1.4.5 SLS (Selective Laser Sintering)

This technique is very important in 3D printing because it allows to print the overhanging parts of the structures without the utilize of supports, in this way it is possible to design and obtain complex structures without the problem of supports removal. It utilizes a CO_2 laser which fuse together the powder contained in a reservoir layer by layer, following the pattern obtained from the sliced pieces of the designed structure; an amount of powder is added at the beginning of each layer, thus it is stacked layer by layer even if it is not fuse together and this cause that the powder itself act as a support for the overhanging parts of the designed structure (Figure 1.19) [10] [30].

Selective laser sintering is a technique utilized in scaffold fabrication for bone tissue engineering due to its possibility to fuse together powder or micro-spheres of hydroxyapatite (HA) and polymers such as polycaprolactone (PCL) which are able to simulate Young's modulus of bone and to stimulate its regeneration [30]. Until now there have not been works which utilized this kind of technique for scaffold vascularization but due to its main advantages, previously exposed, it is good candidate to print a sacrificial vascular network, in particular through the utilize of PVA powders which as mentioned in the work of K.H. Tan et al. [31] can be printed through 3D printers which utilize SLS technique by changing the parameters of commercially available printers.

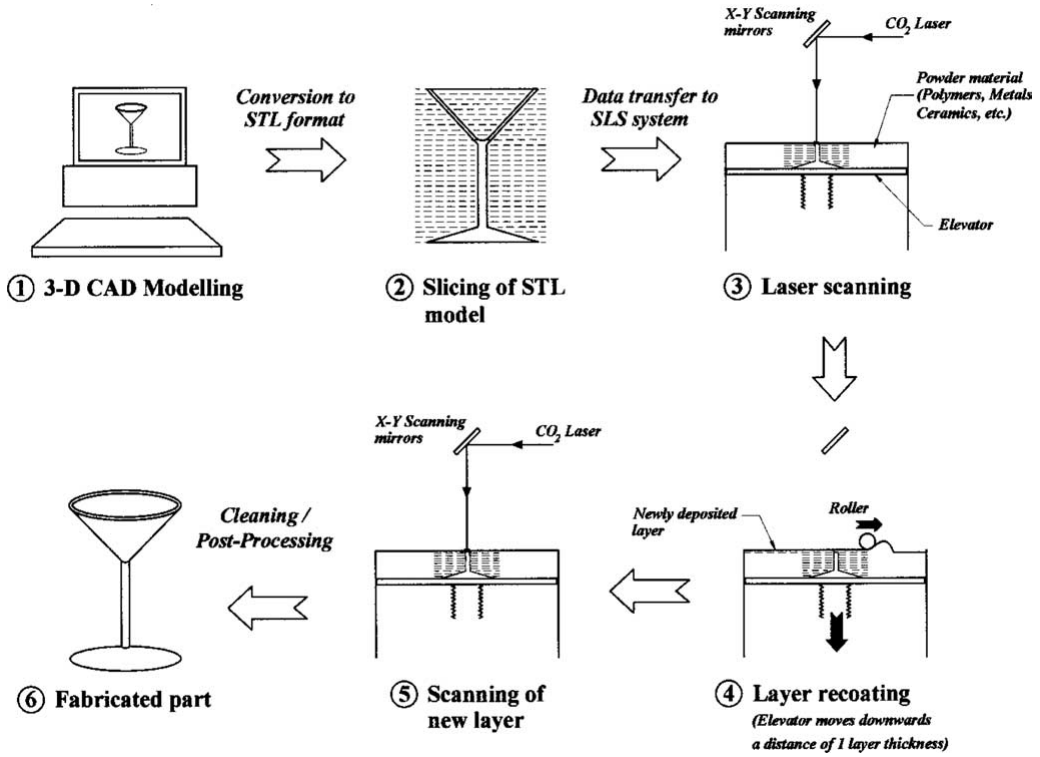


Figure 1.19: *In the figure it is shown the procedure followed with a 3D printer which utilize SLS technique to obtain a designed structure [31].*

1.4.6 Bioprinting of cells

In the last years several researchers have been developed ways to 3D printing directly cells, in this way it is possible to print cells directly on the scaffold and not to seed them on this one once it is obtained. These techniques utilize *cell-loaded bioinks* which are hydrogels solutions containing cells, furthermore there are other kind of bioinks utilized in bioprinting such as: *cell-free bioinks* which are made only by hydrogels solutions and have a support function, *cell-only bioinks* which consist in solutions of cells but they are not too much utilized because cells once printed request a support to maintain their organization [25].

This paragraph is focused on the techniques which allow to print directly cells because other kind of bioprinting (such as: FDM, SLA and DLP commercially available and modified to obtain a 3D bioprinting technology) have already been

exposed in the previously paragraphs.

The main methods which allow to print directly cells are: *Inkjet bioprinting*, *Extrusion bioprinting* and *Laser induced forward transfer*. These techniques use the same technology of 3D printing to generate the designed or acquired (through medical images) structure: the CAD drawing is sliced through a slicing software and this one produce a g-code which is utilized by the printer to print the desired structure layer by layer [28].

Inkjet bioprinting (Figure 1.20) is a technique which utilize a thermal or piezoelectric actuator to squeeze the printer head and generate droplets of cell-loaded bioink stored into the ink cartridge. This technique have the following advantages: it is low cost, it posses a high printing speed and can maintain a high cell viability; whereas its disadvantages are: risk of head clogging if the bioink viscosity is too high (which happens when the hydrogel solution is too viscous or when there is a bioink with high cell density), during the printing process cells settle in the cartridge and can clogging the printer head [28] [10] [32].

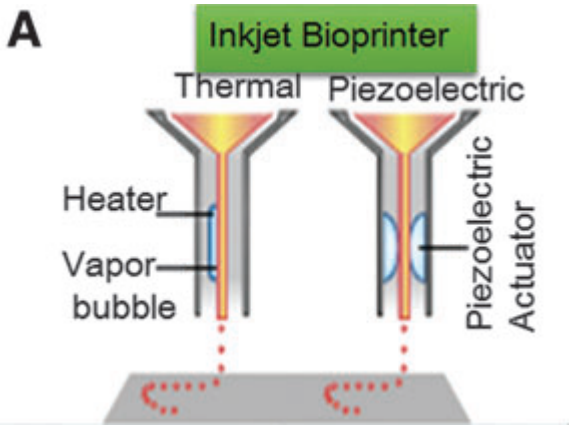


Figure 1.20: *The figure shows the two types of inkjet bioprinters: thermal and piezoelectric. As it possible to see the ejected bioink forms droplets in both cases[33].*

Laser induced forward transfer (Figure 1.21) utilizes a laser which act on a structure composed by an energy-absorbing layer on the top and a bioink layer on the bottom. The heat produced by laser on the energy-absorbing layer create an air

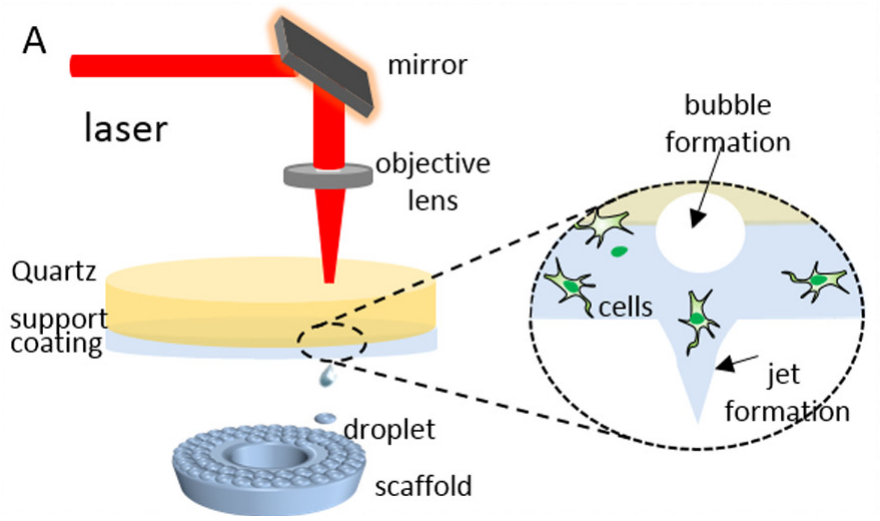


Figure 1.21: *In the figure is showed the process utilized by a laser induced forward transfer technique to print droplet of bioink [32].*

bubble which push the hydrogel cell laden solution of the bottom layer downwards, in this way the fallen bioink droplet is collected on the receiving substrate and then crosslinked. This technique do not cause a mechanical stress on the cells and this increments the cell viability respect to inkjet bioprinting, furthermore also high viscous hydrogels can be printed with it and so more bioinks than in Inkjet bioprinting can be used. The drawbacks of Laser induced forward transfer are: high equipment cost and the fact that laser effects on cells have not been fully understood yet [28] [10] [32].

Extrusion bioprinting (Figure 1.22) is an alternative to inkjet bioprinting which allow to print bioinks contained in a cartridge by pushing them through a nozzle utilizing a pneumatic pump or a mechanical plunger. This technique allow to print cylindrical lines of bioink by applying a continuous force instead of droplets, whereas inkjet bioprinting and laser induced forward transfer allow to print only bioink droplets. The main advantage of this technique is the possibility to print a large amount of hydrogel solutions also with high viscosities and high cell densities but their main drawback is to expose cells to high mechanical stress[28] [10] [32].

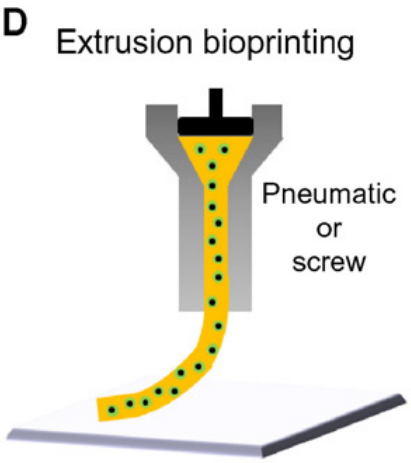


Figure 1.22: *This figure shows how the extrusion bioprinting works. The method through which the bioink is ejected can be pneumatic or mechanical through the utilize of a screw [28].*

Vivian K.Lee et al. [34] utilized an inkjet bioprinter in order to obtain a channel embedded in a collagen matrix. The channel was printed with gelatin which contained human umbilical vein endothelial cells (HUVECs) and then once both collagen and gelatin were solidified this last one was liquefied, in this way the endothelial cells seeded the inner of the channel left in collagen matrix by the gelatin removal (Figure 1.23). In this case the utilize of a bioprinting technology allow to prevent the passage in which cells are seeded in the channels obtained in the scaffolds because this passage is performed during the gelatin removal and this is better when dealing with more complex vascular network. They obtained elliptical channel ($0.7 - 1.5mm$ of width and $0.5 - 1.2mm$ of height) due to the inkjet bioprinting process which print droplets but this was not a problem because, as previously exposed in works which involve microfabrication techniques, endothelial cells tends to remodel the channel shape changing it into a circular one [23] [7].

Another relevant work is the one performed by David B. Kolesky et al. [35]. In this work the authors utilized a custom designed extrusion bioprinter to print

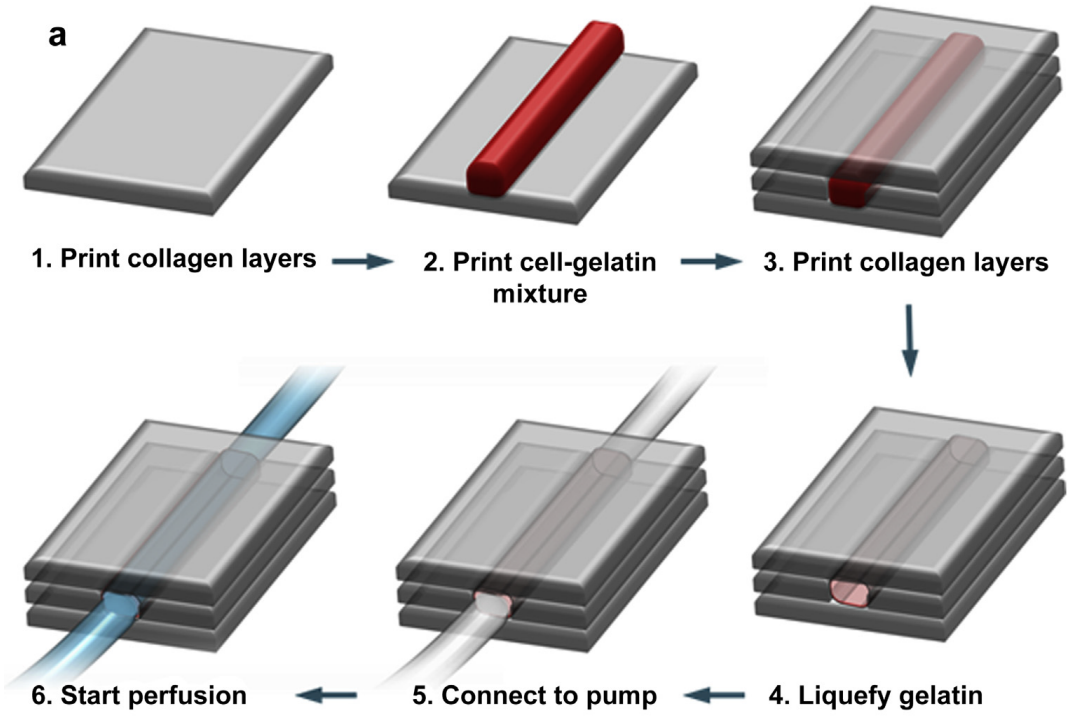
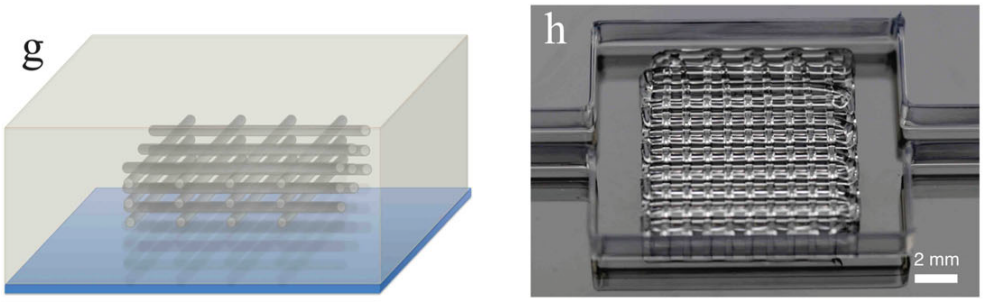


Figure 1.23: *In the figure is shown the process utilized by Vivian K. Lee et al. to obtain a channel in a scaffold made by collagen utilizing an inkjet bioprinter with cell-laden bioink (gelatin and HUVECs) and cell-free bioink (collagen) [34].*

cell-free or cell-loaded bioinks in order to obtain vascularized tissues. The engineered tissues obtained were made by gelatin methacrylate (GelMA) with or without cells (which can be cross-linked through UV light due to methacrylate presence in gelatin) and Pluronic F127 used as sacrificial structure to obtain the vascular network. The vascularization is obtained by liquefying the vascular network printed with Pluronic F127, which is possible by reducing the temperature of the printed structure below its critical temperature having a micelle concentration above the CMC (critical micelle concentration). The printing procedure is performed through printer heads which have $200\mu m$ of nozzle diameter, at a temperature of $20-22°c$ at which both GelMA and Pluronic F127 are in gel state. On the structures obtained they deposited GelMA without cells at $37°c$ to cover the eventually empty spaces left by the printing process and then UV light is



(a) *(b)*

Figure 1.24: *The figure shows: (a) the schematic representation of a 3D vascularized scaffold designed by David B. Kolesky et al. and (b) the final structure obtained by them through their custom made extrusion bioprinter [35].*

applied to cross-link GelMA. The channels obtained in the scaffold composed by GelMA and cells were endothelialized and perfused. The authors were able to obtain channels with a diameter of $100\mu m$ and even less; furthermore they reported that, after printing, channels of Pluronic F127 swell due to the fact that water pass from GelMA to them and this can increment their diameter until the double of the ones printed. Finally they evidence a decreased cell viability in the initial period, probably due to the shear or extensional stress at which cells undergo in the printing process. In the Figure 1.24 it is possible to see an example of a three dimensional vascular network that the authors obtained in a GelMA scaffold through extrusion bioprinting.

These kind of techniques can allow to obtain vascularized scaffolds preventing some passages which are requested with other methods, however they have some limitations such as: the minor diameter reached with these techniques is approximately $150\mu m$ (the medium capillary diameter is $8\mu m$), high time requested for the print, complexity in the choice of the adequate materials which need to: maintain cell viability, structural integrity of the structure and finally to cross-link rapidly after printed [10].

Chapter 2

Materials and methods

2.1 Procedure designed to vascularize a scaffold

The aim of this work is the vascularization of a scaffold in order to allow the survival of cells present in the scaffold core. To reach this goal it was designed the following procedure (Figure 2.1) inspired by a precedent work performed by Rodrigo Pimentel C. et al. [17]:

1. Design and fabrication of a hydro-soluble vascular network with more than one plane through a double extruder FDM 3D printer: supports are printed in PLA whereas the vascular network structure in PVA.

2. Dissolution of PLA supports through immersion of the entire structure obtained with the 3D printer in chloroform.

3. Design and fabrication of the scaffold mold which will contain the PVA vascular network structure during its encapsulation within the hydrogel.

4. Encapsulation of the vascular network in a hydrogel, controlling the gelation time to prevent PVA dissolution before it is completed, dosing the reagents quantity in order to give it mechanical properties similar to the tissue of application and keep it enough rigid to prevent the collapse of vascular network formed once the PVA is eliminated.

5. Active perfusion of the PVA vascular network encapsulated in the hydrogel through an hydraulic circuit using a peristaltic pump to remove the PVA structure and allowing the vascular network formation within the hydrogel.

6. If it is necessary, perfusion of the vascular network with a cross linking agent in order to consolidate the formation of channels within the hydrogel and application of the same solution also on the hydrogel surface to improve its reticulation.

In a previously thesis work by Fabio Chiodini [36] it was created a prevascularized scaffold with a 3D FDM printer following biomimetic considerations but with the vascular network obtained still remained some spaces without a correct vascularization in the scaffold core, thus in this work the aim is not to obtain a vascular network as much as possible biomimetic, but try to guarantee the correct supply of oxygen and nutrients to all the cells present in the scaffold core. Furthermore the present thesis work it is changed the way through which the vascularization is performed: it is obtained directly within the hydrogel through dissolution of a PVA structure and not with a PVA network which act as a mold for silicon rubber (Silplay 184/28) to obtain a structure which then is embedded in the hydrogel. Another innovation designed in this work consists in the realization of a vascular network on multiple planes and this is possible through the utilize of a double extruder 3D FDM printer which allow to print two different types of materials for supports and designed structure simultaneously, in this way it is possible to remove easily the supports by dissolve them into a chemical solvent which do not attack the designed structure. This procedure is necessary in order to obtain a vascular network on multiple planes because, with a single extruder 3D FDM printer, supports are difficult to be removed for the following reasons: they have dimensions similar to the structure and the same color of it, they are difficult to reach manually and finally with a manual removal there is the risk to break the designed structure.

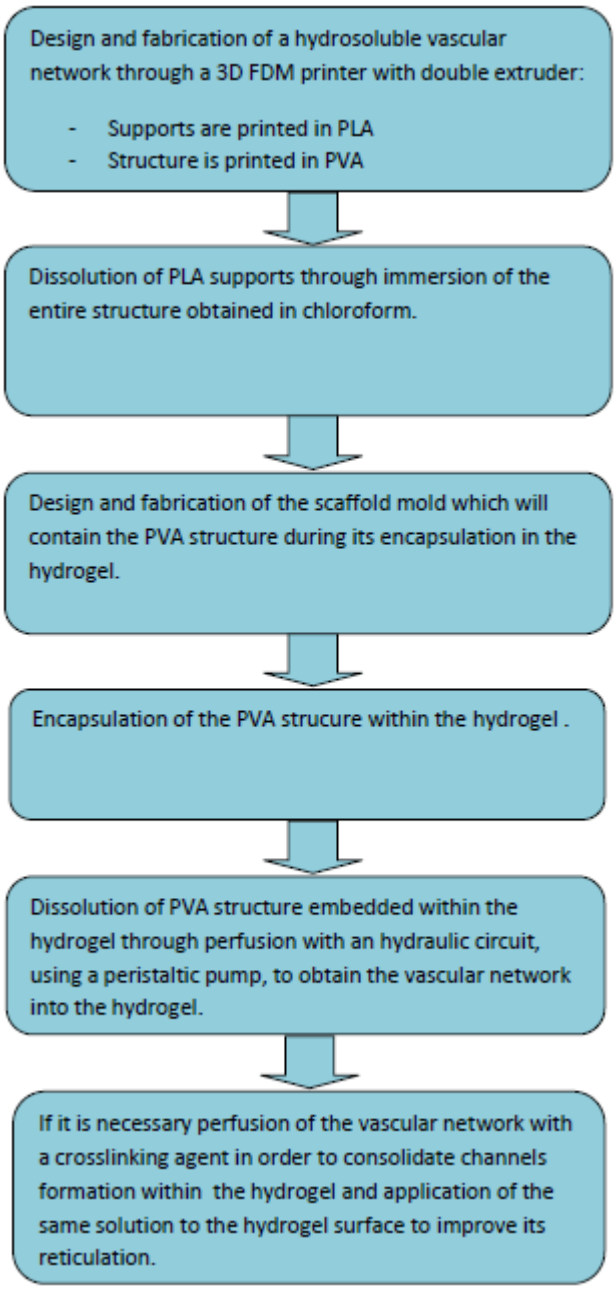


Figure 2.1: *Scheme of the procedure designed to vascularize a scaffold.*

2.2 Design and fabrication of hydro-soluble vascular network

The goal in design of hydro-soluble vascular network was to fill an area and then a volume through an hydraulic network following an approach which is not biomimetic with a the native vasculature but that consider the best way to ensure an adequate supply of nutrients and oxygen to all the cells present in scaffold core. This approach is based on the following prescriptive specifics:

- *Keep the vessels at a maximum distance between them of* $200\mu m$ - This is due to the fact that in native vasculature, capillaries, are at a maximum distance of $200\mu m$ between each other which is correlate to the diffusion limit of oxygen [9].

- *The vascular network must have only 1 inlet and 1 outlet* - In case of more than 1 inlet and 1 outlet the hydraulic circuit will be more complicated to be perfused, furthermore in successive clinical developments of this procedure it will be possible to connect inlet and outlet to the patient's circulatory system.

The desirable specifics are:

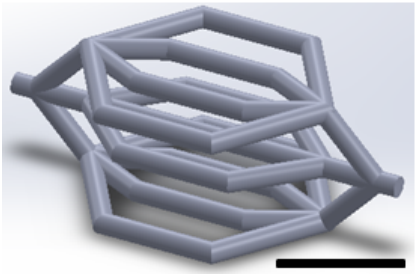
- *Simplifying avoiding too much branches.*

- *Keep the same pattern for the branches* - In this way the fluid will flow with a similar flow rate in all the branches.

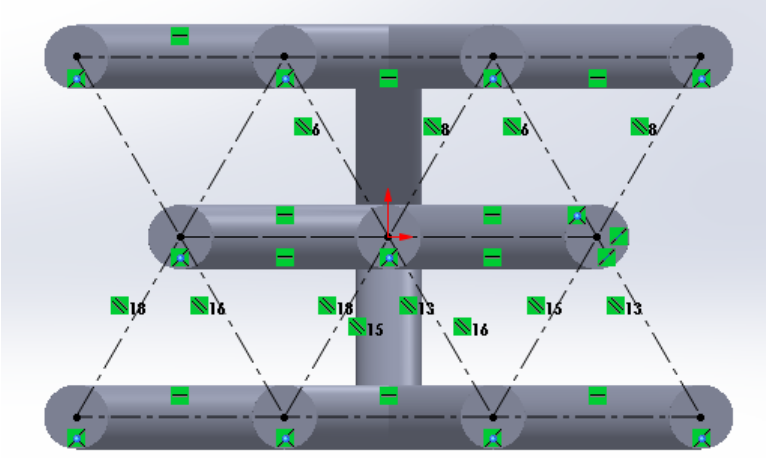
- *Using Murray's law* - Murray's law allow to keep a constant shear stress during branching [37], otherwise it is necessary to calculate the shear stress in the most critical channel in order to verify that, with the flow rate applied, it is lower than $1Pa$ which is the limit tolerable by the cells: over this limit they are detached from channel walls.

All the drawings are obtained through Solidworks, which is a Computer Aided Design (CAD) software.

The first vascular network model (Figure 2.2a) was designed for simplicity without Murray's law, with channels diameter constant in all the network and 10 times bigger than the one of capillaries (capillaries medium diameter is between $5-20\mu m$ [7] and the one chosen in this drawing is $0.09mm$), with 3 planes and considering a distance between each channels of $200\mu m$ [9] which led to consider each channel in the lateral section of the structure central zone as a vertex of an equilateral triangle as can be seen in the Figure 2.2b.



(a)



(b)

Figure 2.2: *(a) Drawing of the first designed vascular network using dimensions as similar as possible to capillaries without considering Murray's law (scale bar:* $0.55mm$*). (b) Lateral section in the central zone of the first designed vascular network. It is possible to see the equilateral triangles that, with their sides length (comprising the channel radius), set the distance between channels at* $200\mu m$*.*

An equilateral triangle have all the sides with the same length and all the angles equal to 60°, thus the distance between central plane and superior, which pass through the channels centers, is calculated through this equation:

$$h = a * \sin 60° \tag{2.1}$$

which calculate the height of an equilateral triangle where "a" is the side of the equilateral triangle and in this case it is calculated through this equation:

$$a = 0.2mm + \frac{central\ channels\ diameter}{2} + \frac{superior\ channels\ diameter}{2} \tag{2.2}$$

In equation 2.2 the value 0.2mm is referred to the distance between each channel in the space.

In Figure 2.3 it is possible to see the geometry connected to these equations for the superior plane (the inferior plane is obtained through symmetry performed with respect to the central plane).

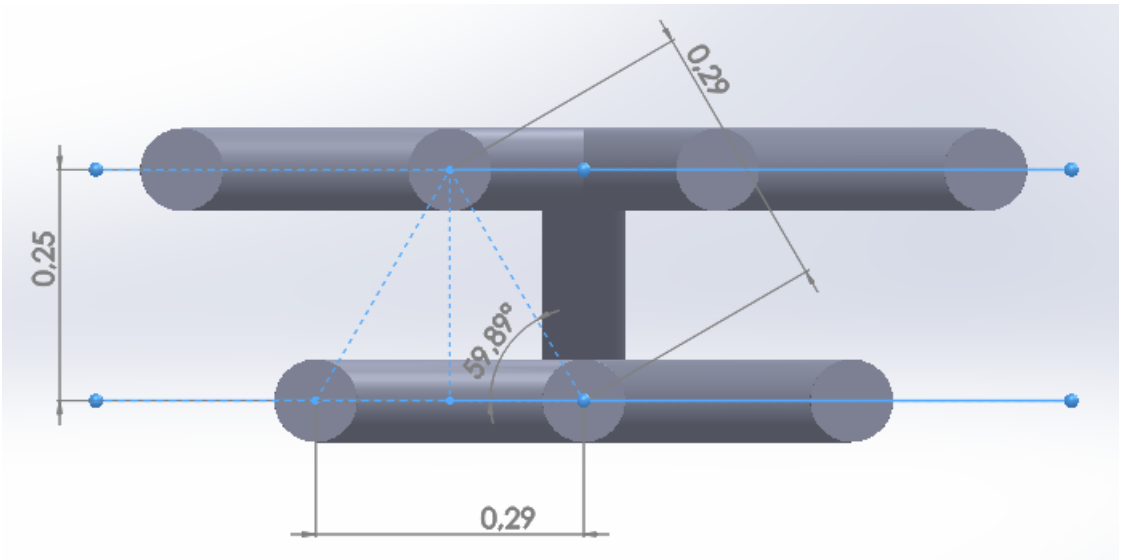


Figure 2.3: *Lateral section of vascular network central zone (in this case channels diameter is 0.09mm and it is not considered Murray's law). It is possible to see one of the equilateral triangles which set the distance between channels in the central zone with the dimensions of its sides* $a = 0.29mm$ *and its height* $h = 0.25mm$.

The small dimensions of the designed vascular network do not allow to print it with a 3D FDM printer neither with a 3D SLS printer with a laser diameter about $300\mu m$, thus its dimension were changed in order to print it with a 3D SLS printer because it request an increasing in the designed dimensions lower than a FDM 3D printer with a nozzle diameter of $400\mu m$ like the one of the printer present in LaBS. The channel diameter was increased of other 10 times (reaching a diameter of $0.9mm$) and the distance between each tube was changed to be $400\mu m$. In this way the 3D printing service company: "ABprint3D" was able to print the structure with nylon powder (Figure 2.4) without any support (this was possible due to the utilize of a 3D SLS printer).

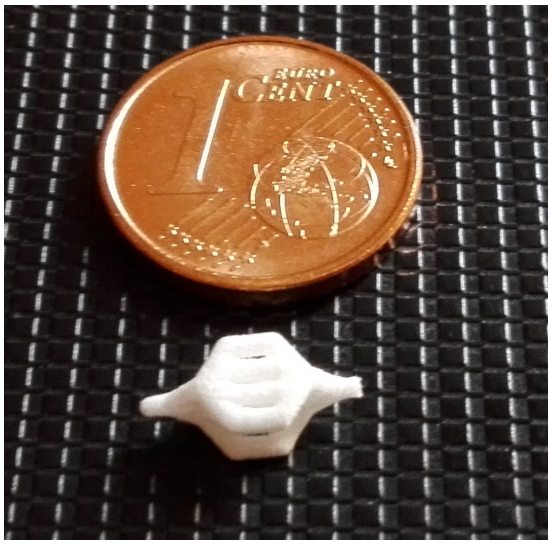


Figure 2.4: *Vascular network printed through a 3D SLS printer by the 3D printing company: "ABprint3D".*

Unfortunately nylon powder is not a material which can be dissolved through water like PVA but there are ways to print PVA powder [10, 31], even if the 3D printing companies generally do not it because PVA powder is too expensive and the slice of market which request it is too small.

In the present thesis work is utilized the 3D printer available in LaBS (Laboratory of Biological Structure Mechanics) which is a fused deposition modeling (FDM)

printer and so utilize filaments of thermoplastic materials such as PLA (polylactic acid), ABS (acrylonitrile-butadiene-styrene), PVA (polyvinyl alcohol) and other commercial filaments (Figure 2.5). This 3D printer have only one extruder and so allow to print only vascular network with one plane for the reasons exposed in the previous paragraphs; it is a reproduction of the "original Prusa i3", thus it works with the slicing software associated to this printer: "Ultimaker Cura".

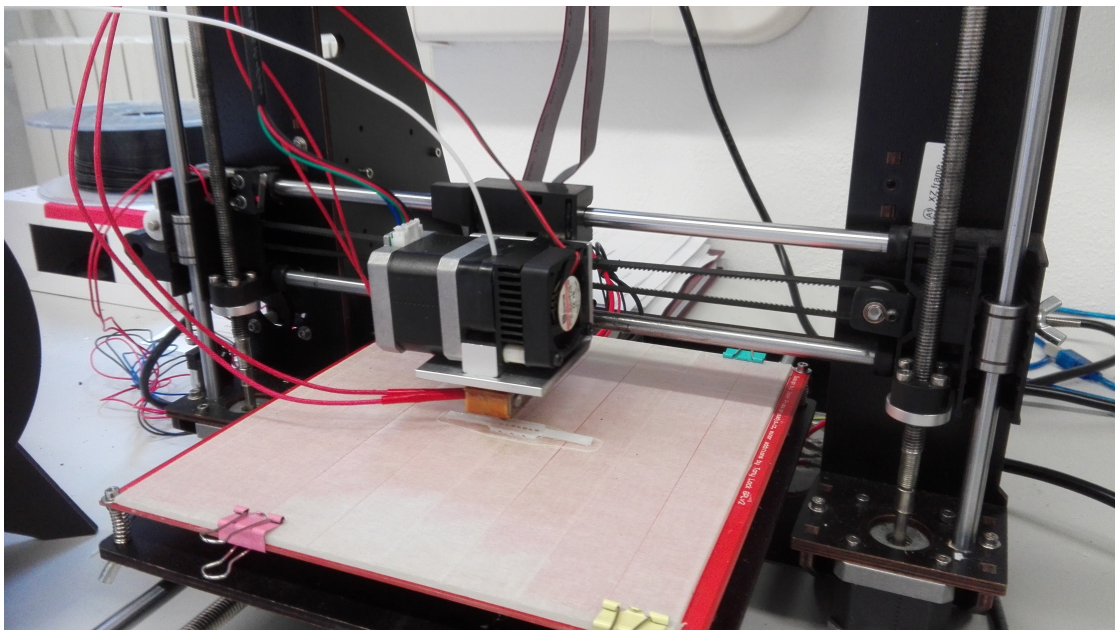
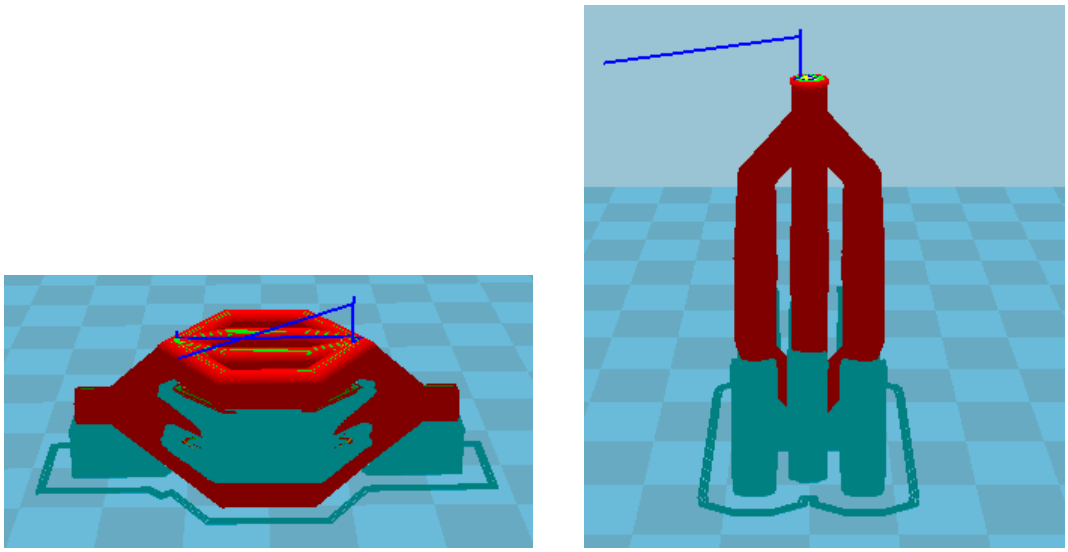


Figure 2.5: *3D printer present in LaBS.*

In order to utilize a 3D printer it is necessary: design the drawing through a CAD software (in this thesis work it was utilized Solidworks), save the drawing with an "stl" extension and finally utilize a slicing software (in this case it was utilized Ultimaker Cura) which through algorithms slices the drawing with "stl" extension in a series of layers and inserts the supports necessary to print suspended parts. On slicing software it is possible to choose the settings to control 3D printer such as: plate adhesion, supports, printing speed, material utilized, layers thickness, ... and these information are transmitted to the 3D printer through the generation of a G-code file.

The 3D printer present in LaBS was utilized to print the modified model previously printed by the 3D printing company: "ABprint3D" but its dimensions were increased of 500% to allow the printing through this 3D printer. The Figures 2.6a and 2.6b are captured from the software "Cura" and allow to see the position of supports generated when the designed vascular network is printed respectively horizontally and vertically.



(a) *(b)*

Figure 2.6: *Pictures captured from the software "Cura" which show the position of supports generated by the slicing software for the designed vascular network with dimensions modified and increased to be printed with 3D FDM printer present in LaBS, in the cases of it will be printed horizontally (a) or vertically (b). In the figure supports are colored in light blue whereas the structure is red.*

Apparently seems better to print the vascular network vertically to have less difficult in supports removal but if the vascular network is printed in this way the 3D printer produce a non linear profile of the vessels which do not happen if it is printed horizontally, thus the choice was to print it horizontally. In the Figure 2.7 is possible to see the supports printed with the same material of the structure which partially fuse with it and occupy the small space between the planes preventing their manual removal.

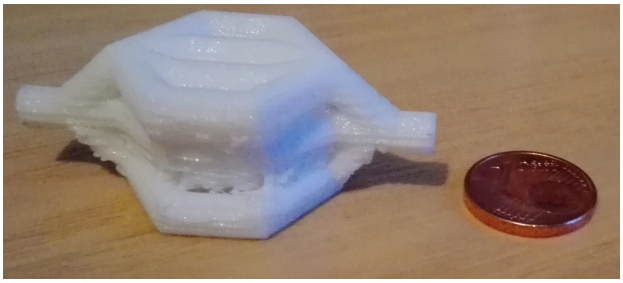


Figure 2.7: *Model printed in PLA through the 3D printer present in LaBS with dimensions increased of 500% respect to the one printed by 3D printing company: "ABprint3D" which was obtained in nylon, through a 3D SLS printer.*

Furthermore the drawing of the first vascular network model was implemented considering Murray's law:

$$d_p^3 = d_{s1}^3 + d_{s2}^3 + ... + d_{sn}^3 \tag{2.3}$$

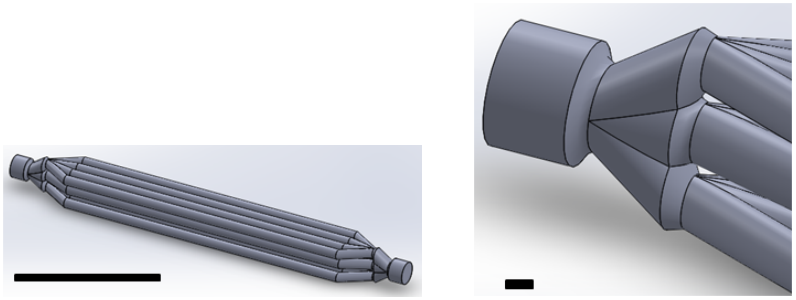
which means that the cubic diameter of parent channel is equal to the sum of son channels cubic diameters, assuming an axisymmetric geometry ($d_{s1} = d_{s2} = d_{s3}$ and $l_{s1} = l_{s2} = l_{s3}$, d=diameter, l=length) this equation can be written as

$$d_p^3 = n * d_s^3 \tag{2.4}$$

where n=number of son channels [37]. Thus diameter of each parent channel is reduced at each branch of this entity:

$$d_s = \frac{d_p}{\sqrt[3]{n}} \tag{2.5}$$

through a connection element which is possible to see in the Figure 2.8b.
Three drawings have been obtained of the same model with the implementation of Murray's law and different inlet/outlet diameter which have led to different channels diameter. These three drawings utilize respectively the following measures: inlet/outlet channels (1mm, 1.5mm, 2mm), channels which conduct to each plane (0.69mm, 1.04mm, 1.38mm), channels of the middle plane (0.47mm, 0.72mm, 0.95mm), channels of the superior and inferior planes (0.43mm, 0.65mm,



(a) *(b)*

Figure 2.8: *(a) Drawing of the vascular network model with the implementation of Murray's law (inlet/outlet diameter=1.5mm) (scale bar: 10 mm). (b) View of the connection elements present in the ramifications which allow to connect channels of different diameter (inlet/outlet diameter=1.5mm) (scale bar: 0.2 mm).*

0.86mm). The total number of the channels was 19. In these vascular networks, which consider Murray's law, it was not possible to maintain the equilateral triangles which set the distance between each channel in the central zone of the structure due to the differences in channel diameters (see the Figure 2.9).

Considering drawings with different channel diameters is essential to find the smaller dimensions printable in order to reach values as similar as possible to the ones of capillaries.

The same vascular network was designed without considering Murray's law (same channel diameter in all the structure) for diameters of: $2mm$, $1.5mm$ and $1mm$; this was performed in order to increase the small vessels dimension obtained with the first drawing to allow the printing through a 3D printer which utilize the FDM technique. In these drawings it was possible to maintain the equilateral triangles which define the distances between channels in the lateral section of the vascular network central zone.

Furthermore it was supposed the utilize of a square instead of a triangle geometry in the vascular network central zone (Figure 2.10) to define the distance between each channel in order to simplify the fluid dynamics of the structure but in this way, along the squares diagonals, channels are not separated by a distance of

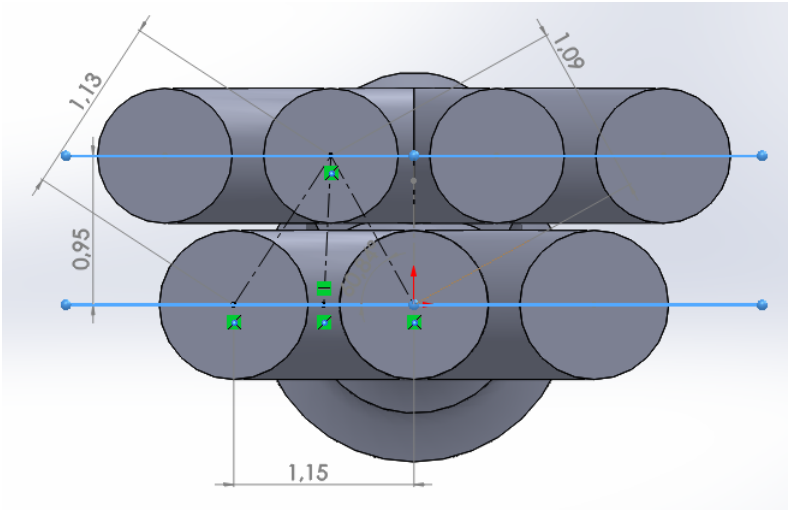


Figure 2.9: *Lateral section of vascular network central zone (in this case inlet channel diameter is 2mm and it is considered Murray's law which lead to 0.95mm of middle plane channels diameters and 0.86mm of superior plane channels diameter). It is possible to see that triangles are no more equilateral due to the different channel diameter between channels of middle and superior planes.*

$200\mu m$ in fact the square diagonal is defined as:

$$d = \sqrt{2} * a \tag{2.6}$$

Where "a" is the value of square side and it is equal to:

$$a = 0.2mm + 2 * channel \quad radius \tag{2.7}$$

Thus, along square diagonal, the distance between channels is incremented by a factor of $\sqrt{2} = 1.4$ whereas using equilateral triangles their sides values define the distance between channels in the lateral section of vascular network central zone. For this reason it was decided to set the distance between channels in the vascular network central zone through equilateral triangles.

Finally between the several vascular networks designed, the chosen one, to be obtained through a 3D FDM printer with double extruder, was that with the following characteristics (Figure 2.11):

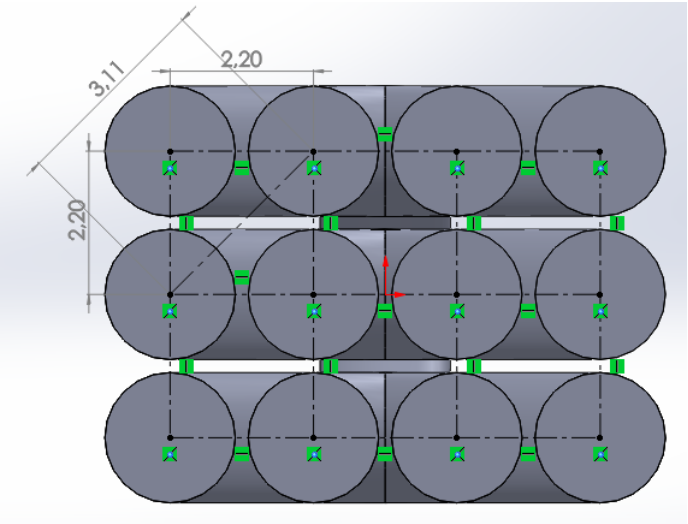
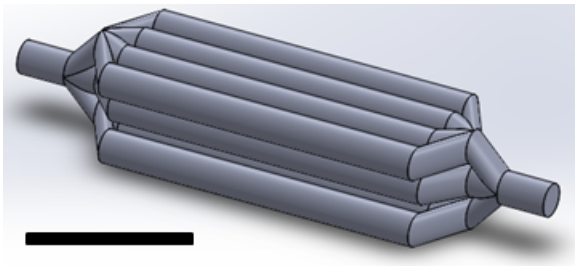
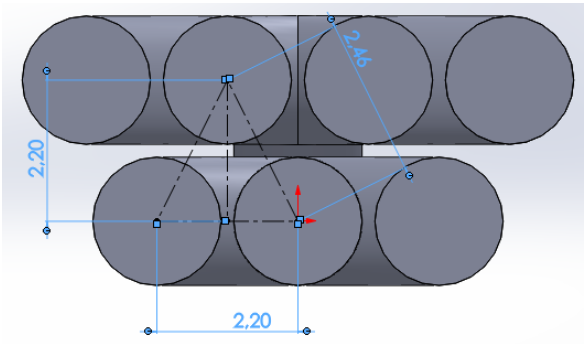


Figure 2.10: *Lateral section of vascular network central zone in which the distance between channels is imposed by the sides of squares. As it is possible to see along diagonals the distance between channels increase respect to their sides.*

- Diameter of the vessels equal to $2mm$.
- Distance between channels, in the central zone of the same plane, set at $200\mu m$ but the height of the equilateral triangles which set the distances also between different planes was increased of $0.3mm$ respect to the one calculated through equation 2.2 and 2.1 to increment the capacity of the designed vascular network to be printed through a FDM 3D printer with nozzle diameter of $0.2mm$ and $0.5mm$ of layer thickness which are the smallest dimensions commercially available for this kind of printer; thus the triangles are no more equilateral.
- Do not follow Murray's law because this simplify the fluid dynamics of the vascular network and the realization of its details through a FDM 3D printer. In fact the major advantage of Murray's law consist in keeping constant shear stress along the network but as it is possible to see in the next paragraph, it can be maintained very lower than the critical value at which cells detach from channel walls ($1Pa$).



(a)



(b)

Figure 2.11: *(a) Drawing of the chosen vascular network between the ones designed (scale bar:10 mm). (b) Lateral view of the central zone of vascular network chosen. It is possible to see that the equilateral triangles, which set the distance between the channels, are no more equilateral, im fact their height was increased to improve the capacity of the vascular network to be printed.*

2.3 Fluid dynamics of vascular network

It is necessary to define the flow rate through which the vascular network will be perfused by a peristaltic pump. In order to do this it is calculated the maximum value that the flow rate can have because over this it is caused a shear stress above the admissible threshold, this maximum value is calculated in the most critical case which corresponds to the channel with minor diameter (in the final design chosen for the vascular network all the channels diameters are equal and correspond to $2mm$).

Firstly it is necessary to find the expression which binds flow rate with shear stress in order to obtain the maximum flow rate through which it is possible to perfuse the channel network without reaching maximum shear stress ($\tau < 1Pa$, [6]) that

would cause the removal of endothelial cells which will be seeded on channel walls. Considering a rectilinear cylindrical conduit (Figure 2.12) the equilibrium of forces

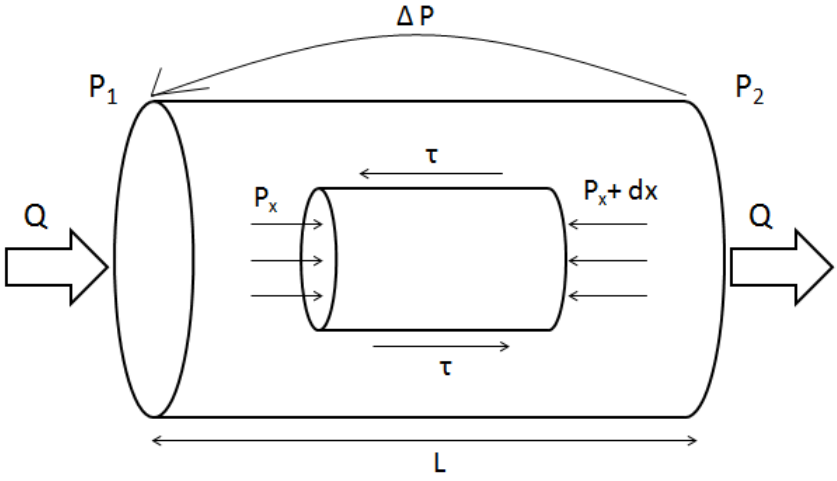


Figure 2.12: *Schematic representation of a rectilinear cylindrical conduit and a coaxial cylinder of fluid with the forces which act on this last one.*

which act on a coaxial cylinder of fluid respect to the symmetry axis is:

$$F_x + F_P - F_\tau = 0 \tag{2.8}$$

Where: $F_x = ma_x$ is the force due to the acceleration of fluid which is null because the flow is completely developed, $F_P = (P_1 - P_2)\pi r^2$ is the force due to the pressure difference and $F_\tau = \tau 2\pi rl$ is the force due to the shear stress, so the equation becomes:

$$(P_1 - P_2)\pi r^2 - \tau 2\pi rl = 0 \tag{2.9}$$

From the equation 2.9 can be obtained:

$$\Delta P = \frac{2\tau l}{r} = \frac{4\tau_P l}{D} \tag{2.10}$$

The Poiseuille's law is defined as:

$$Q = \frac{\pi D^4 \Delta P}{128\mu l} \tag{2.11}$$

Equation 2.10 can be substituted into Poiseuille's law to obtain the expression which bind flow rate to wall shear stress:

$$Q = \frac{\pi D^3 \tau_P}{32\mu} \tag{2.12}$$

Where: $Q = flow\,rate$, $D = channel\,diameter$, $\tau_P = wall\,shear\,stress$, $\mu = viscosity$.

Considering that: all the channels in the network have a diameter of $2mm$, water viscosity is $1cP = 0.001Pa * s$ (the culture medium is an aqueous solution so it is possible to consider it as water) and the maximum wall shear stress have to be lower than $0.5Pa$ to maintain a security coefficient of 2 respect to the critical value of $1Pa$ it is possible to calculate that the maximum flow rate to prevent cell detachment in the designed vascular network is $23.52\frac{ml}{min}$.

In order to choose an adequate flow rate to perfuse the vascular network once the PVA is dissolved it was supposed to utilize a flow rate which allow to have a physiological velocity in the central zone of vascular network where the channels are set to be at physiological distance ($200\mu m$ [9]). In this way it was supposed that the delivery of solutes to cells present in the hydrogel and removal of waste products is adequate but it is also necessary to consider that in the hydrogel there is a solutes depletion not only along the radial direction but also along the axial one and this can cause that cells present within hydrogel in vascular network central zone near outlet receive lesser oxygen and nutrients than the ones near inlet; it is necessary to assure that these cells will receive an adequate amount of solutes which is important for their survival and this request a practical evaluation and mass transport calculations of all solutes which here are not performed but can be discussed in successive studies. The present thesis work choose as length for the vascular network central zone to utilize an arbitrary value to allow the vascular network manual manageability ($20mm$) and do not focus on solutes depletion along this length.

The average velocity through which blood flows in capillaries is comprise between

$0,1\frac{mm}{s}$ and $0,5\frac{mm}{s}$, these values are approximations obtained by two books: "Fisiologia" [21] and "Testo Atlante di Fisiologia Umana: Apparato Cardiovascolare" [38]. The flow rate necessary to obtain a culture medium velocity in the central zone channels which is comprised in these values was calculated with a finite volume simulation software (Ansys Fluent).

In order to simulate the fluid dynamics of culture medium in the designed vascular network this last one was cut in the half of its length to consider as outlets all the channels present in central zone (Figure 2.13) and through the information of mass flow provided by the software their velocities were calculated. The simulation software allow to set: steady state conditions, gravity field, water ($\rho = 1000\frac{kg}{m^3}$, $\mu = 1cP = 0,001\frac{kg}{ms}$) as fluid which flows in the network (the real fluid is a culture medium but it is an aqueous solution, thus it can be approximated as water), a laminar flow, pressure at the outlets ($0Pa$ for each outlet) and fluid velocity at the inlet.

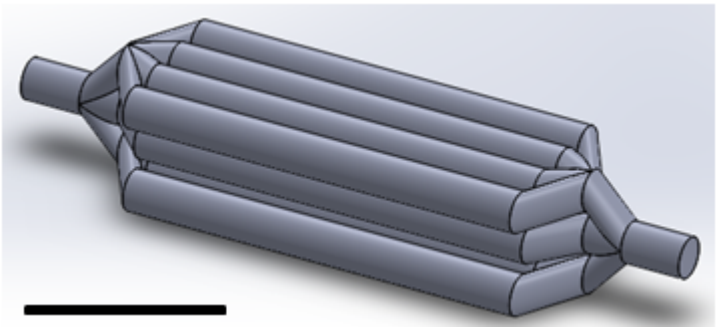
The medium fluid velocity at the inlet is calculated through this equation:

$$v = \frac{Q}{A} \tag{2.13}$$

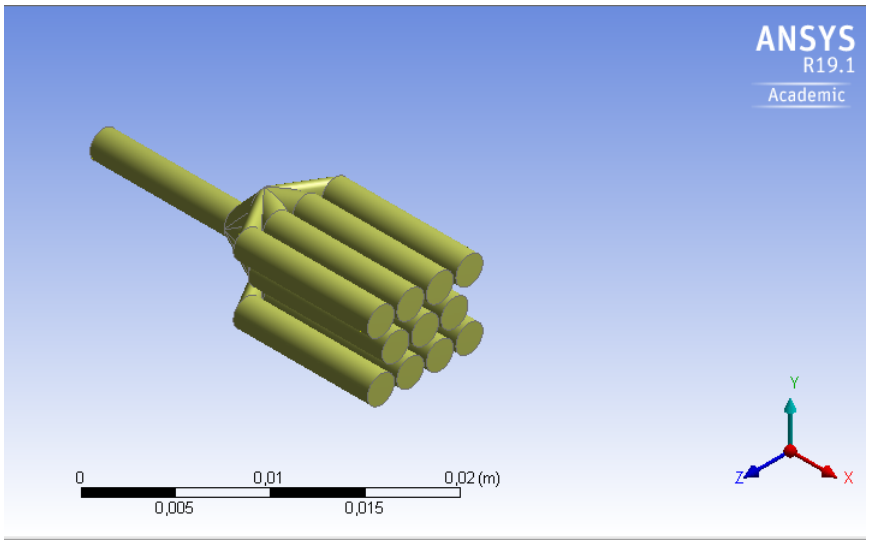
where Q is set as the minimum flow rate which can be imposed through the peristaltic pump that is present in LaBS ($0.8\frac{ml}{min}$), and A is the cross section area, thus the velocity obtained for the inlet is $0.0041\frac{m}{s}$.

The fluid velocity set at the inlet is the average fluid velocity and it was imposed constant along the inlet surface, in fact it was evaluated that after approximately $3.5D$ (where D is the channel diameter) the velocity profile along y and z direction becomes a parabola for a fluid moving in x direction, thus the inlet was elongated of $3.5D$ respect to the real design (Figure 2.13), in this way it is not necessary to utilize an user defined function (udf) which set automatically the right velocity profile at the inlet. It was supposed that the flow is a laminar flow and then it was evaluated the truth of this hypothesis. A parabolic velocity profile in both y and z direction is produced for a fluid moving in x direction with a laminar flow

because the fluid sheets slide with respect to each other and near the channel walls their velocity becomes gradually null, in fact fluid sheets in contact with channel walls do not move whereas the velocity in conduit center is two times the medium velocity and this gives origin to the parabolic velocity profile.



(a)



(b)

Figure 2.13: *(a) Original drawing of the vascular network (scale bar: 10 mm). (b) Modified drawing of the vascular network obtained to perform the simulation with Ansys Fluent, it present inlet elongation of* $3.5D$ *and the cut in the half of its length.*

Reynolds number was calculated in order to define if the flow of the fluid which flow in inlet channel is laminar or turbulent and so if the hypothesis previously made of a laminar flow was true:

$$Re = \frac{\rho v D}{\mu} \tag{2.14}$$

Considering water as the fluid which flows in designed channel network its density ($\rho = 1000\frac{kg}{m^3}$), its viscosity ($\mu = 0,001\frac{kg}{ms}$), its velocity ($v_{in} = 0,0041\frac{m}{s}$) and D which is the characteristic length:

$$D = \frac{4 * Area}{Wet\,Perimetry} \tag{2.15}$$

the equation 2.15 in case of a cylindrical channel becomes:

$$D = \frac{4\pi(\frac{D}{2})^2}{2\pi\frac{D}{2}} = channel\,diameter \tag{2.16}$$

and in this case $D = 0,002m$; thus it results that: $Re = 8,2$. This Reynolds number is lesser than 2000, thus the kind of flow through which culture medium flows in the designed network is laminar (Reynolds number was calculated only for the inlet channel because fluid velocity is reduced when channels split and in the equation 2.14 it is the only variable, thus Reynolds number is reduced in the other channels).

It was performed a sensitivity analysis in order to choose the best mesh which can be utilized to perform the simulations considering 5 different meshes and the average percentage error calculated for each mesh used for the simulations with Ansys Fluent. The average percentage error is calculated as the medium of percentage errors obtained for each outlet mass flow rate considering the values obtained with the finest mesh and the values of the current mesh. The finest mesh has 177482 nodes (mesh 5) but it was possible to utilize a mesh with 137898 nodes (mesh 3) in order to reduce the computational time requested to perform the calculations because with this kind of mesh the average percentage error is lesser than 0.5% which was chosen as threshold value to consider reliable the results obtained by performing the simulation with another mesh instead of the finest one. The elements size chosen to obtain the different meshes through the CFD method and the resulted number of nodes are shown in Table 2.1.

All the simulations were performed requesting an absolute convergence for the errors of x, y, z velocity and continuity, imposing a value of: 10^{-5}.

Mesh number	Elements size (m)	Number of nodes
Mesh 1	0,0004	108017
Mesh 2	0,0003	114636
Mesh 3	0,00026	137898
Mesh 4	0,00024	154907
Mesh 5	0,00022	177482

Table 2.1: *Elements size chosen and number of nodes obtained with CFD method (used to perform the meshes) for each mesh considered in sensitivity analysis.*

2.4 Design and fabrication of the scaffold mold

The scaffold mold must be designed considering that it must have small dimensions because the vascular network which occupy its core is designed to ensure the cells survival in this zone and so this imply that is requested the lower possible distance between scaffold core and its borders which allow to maintain its structural integrity and manageability. Following these considerations it was designed the scaffold mold represented in Figure 2.14 adapting it to the dimensions of the printed vascular network with an inlet and an outlet of $2mm$ (the same diameter of the vascular network channels); then the two pieces which would composed the master were designed to obtain it (Figure 2.15).

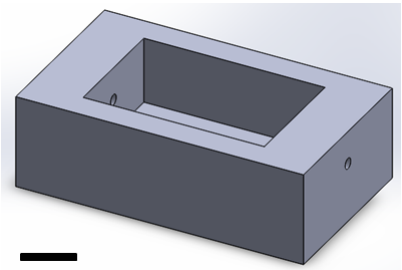
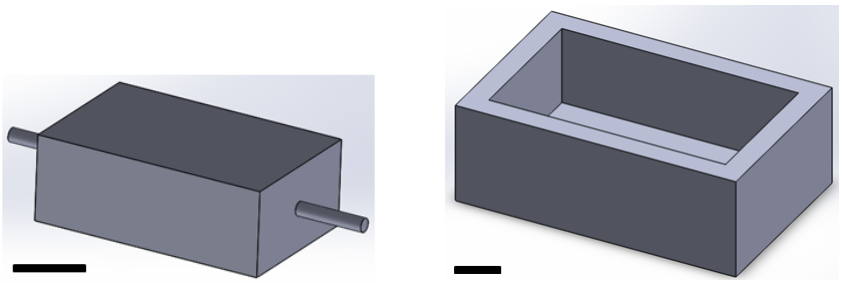


Figure 2.14: *In the picture it is possible to see the designed scaffold mold with a pore of* $2mm$*, where one of the two hydraulic connectors will be inserted (scale bar: 10 mm).*

For the fabrication of the scaffold mold the two pieces which compose the master were printed in ABS through the 3D printer present in LaBS (which utilize FDM technology) and assembled, in this way it was obtained a master in which it was



(a) *(b)*

Figure 2.15: *In the figure it is possible to see the two pieces which compose the master utilized to mold the solution which solidify generating the silicon rubber (Silplay 184/28). Through the insertion of the first piece (a) in the second (b) it is obtained the master (scale bars: 10 mm).*

casted a solution of silicone that once solidified generates a silicone rubber (Silplay 184/28) which can be removed manually from the master and act as a scaffold mold.

The silicone solution is composed by two components: A and B, whose combination at 50:50 concentration allows the solution solidification in approximately one day at environmental temperature. After the silicone solution was casted in the master, this was left in a freezer for 10 hours to remove the air bubbles which can be embedded during the mixing process of the components A and B (during this time the silicone solution do not solidify, the solidification happens only at environmental temperature).

The silicon rubber utilized (Silplay 184/28) is only a food grade material, thus for further developments it is better to substitute it with a biomaterial.

2.5 Hydrogel fabrication

Hydrogels are hydrophilic polymeric networks which can swell without be dissolved in a solvent. They can be obtained from organic or synthetic polymers allowing also hybrid hydrogels and can be crosslinked in several ways, for example: chemical cross-linking, physical cross-linking and cell cross-linking [3] [39]

[40].

The hydrogel utilized in the present thesis work to obtain the scaffold in which is created the vascular network is made by 50% of gelatin and 50% of alginate, thus it is a natural hydrogel because it utilizes two natural polymers, furthermore it uses two physical cross-linking methods to change from solution state to gelation state: thermal cross-linking for the gelatin and ionic cross-linking for the alginate. This kind of hydrogel was chosen prevalently due to: its fast gelation process (approximately 1 hour) which allow to prevent the dissolution of PVA vascular network until the gelation of hydrogel solution is completed, its manageability and also for the presence of gelatin which contains binding sites for cells.

Gelatin is a natural polymer which is obtained by the denaturation of collagen (a natural protein), it is less immunogenic than collagen and it has a thermal gelation which is a quite rapid process when maintained at low temperature. One of the major advantages of gelatin is the fact that it contains RGD sequences (chemical groups composed by three amino-acids: arginine, glycine and aspartic acid) which allow cell adhesion, thus it does not need to be modified in order to allow cell adhesion [39]. The physical gelation of gelatin is performed by dissolving it in water at $40°c$ and leaving the solution obtained in fridge at $4°c$ for approximately half hour; in this way the hydrogel changes from sol state to gel state but this is a reversible transformation, in fact by heating the hydrogel obtained it returns to sol state. In order to increment the stability of gelatin hydrogel and in the meantime maintaining the RGD sequences the solution of gelatin was mixed with another one of alginate in the percentages previously exposed.

Alginate is a natural polymer, it is obtained from brown seaweed and it is an anionic polysaccharide which allow it to be ionically cross-linked in presence of divalent cations such as: Ca^{2+} [41]. Unlike gelatin, alginate do not posses sequences needed for cell adhesion, thus it needs to be chemically modified or combined with other hydrogel in order to allow cell adhesion. Alginate is composed by the fol-

lowing monomers: mannuronic acid and guluronic acid; when it is performed the ionic gelation through solutions that free divalent cations it assume a particular structure which is like to an egg box (Figure 2.16) [40]. The gelation of an alginate

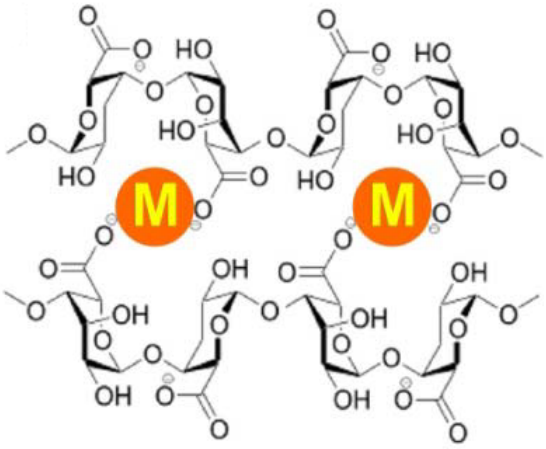


Figure 2.16: *Chemical formula of alginate ionically crosslinked through divalent cations (M in the figure) such as: Ca^{2+}, Mg^{2+} and Fe^{2+}. It is possible to observe the structure similar to an egg box [40].*

solution generally can be performed through the utilize of a $CaCl_2$ solution which release Ca^{2+} in aqueous environment but due to the high solubility of calcium chloride in aqueous solutions, this mechanism has a speed of gelation too fast which makes difficult to control the gel uniformity and strength; thus in order to slow and control the gelation instead of $CaCl_2$ is utilized calcium carbonate ($CaCO_3$) which posses a lower solubility in aqueous solutions [40]. In the present thesis work calcium carbonate is utilized in association with $D-(+)-glucono-\delta-lattone$ (GDL) which allows to dissociate Ca^{2+} ions from calcium carbonate by lowering the PH. Ionically crosslinked alginate hydrogels have a main drawback which is the limited long-term stability in physiological conditions, because these gels can be dissolved due to release of divalent ions in the surrounding environment which is caused by exchange reactions with the monovalent cations present in the environment. This lead to consider the utilize of covalently crosslinked alginate hydrogels through a chemical gelation process which are not studied in the present

thesis work but can be considered in successive studies [41].
In the present thesis work, to obtain the hydrogel with 50% of gelatin and 50% of alginate, was utilized the following procedure previously used in a PHD thesis [42]:

- Preparation of an alginate solution (alginic acid obtained from brown seaweed, A7003, *Sigma Aldrich*) at $8\%\frac{w}{v}$ (weight[g]/volume[ml]).
- Addition of sodium bicarbonate ($NaHCO_3$, S5761, *Sigma Aldrich*) to the alginate solution to reach a concentration of $300mM$ in order to reduce the acidity of the solution.
- Addition of sodium hydroxide ($NaOH$, 71692, *Fluka Analytical*) to the alginate solution to reach a concentration of $40mM$ in order to reduce the acidity of the solution.
- Preparation of a gelatin solution (gelatin obtained from bovine skin, Type B, G9391, *Sigma Aldrich*) at $6\%\frac{w}{v}$ (weight[g]/volume[ml]) in distilled water at $40°c$.
- Mix the two solutions obtained (alginate and gelatin solution).
- Preparation of a solution containing $D-(+)-glucono-\delta-lattone$ (GDL, G4750, *Sigma Aldrich*) at $10\%\frac{w}{v}$ (weight[g]/volume[ml]) in a volume which is the 10% of the total volume of the alginate/gelatin solution.
- Addition of $D-(+)-glucono-\delta-lattone$ solution and calcium carbonate ($CaCO_3$, C4830, *Sigma Aldrich*) to reach a concentration of $50mM$ to the gelatin/alginate solution.
- Pour the solution in the previously obtained mold and keep it at $4°c$ during the reticulation which last for approximately 1 hour.

2.6 Hydrogel mechanical characterization

In a precedent thesis work by Fabio Chiodini [36] were performed mechanical tests on alginate/gelatin hydrogels with different percentage of alginate and gelatin; here are reported the procedures and in chapter 3 the results obtained with these mechanical tests for the alginate/gelatin hydrogel utilized in the present thesis work which is the one with 50% of alginate and 50% of gelatin.
For the mechanical characterization of the hydrogel were performed mechanical tests of non-confined compression with a successive statistic analysis which here is not reported because it was focused on the comparison between the hydrogels with different concentration of alginate and gelatin in order to understand if their different mechanical properties are statistically significant. In the present thesis work the choice of the hydrogel is focused on the gelation time and its manageability, but in lower measure on its mechanical properties, due to the fact that it is not considered a precise tissue of application.
For the hydrogel with 50% of alginate and 50% of gelatin were prepared 4 samples with: cylindrical shape, base diameter $D = 20mm$ and height $h = 10mm$. The mechanical tests were effectuated under *stress relaxation* conditions: each sample undergo deformations of 5%, 10%, 15% and 20% for 2 minutes each one with a ramp of $1\%/s$ as shown in the Figure 2.17.

The stress values where obtained from the force recorded by the software (Win Test 4.1) associated with the test machine (Endura TEC ELF 3200, *BOSE*, with load cell by 22 N); in particular were considered the initial and final stresses of each deformation after the deformation ramp. Furthermore was calculated the elastic modulus for each deformation as the difference between initial stress of the deformation interval i and final stress of the previous interval $i-1$, divided for the incremental deformation $\Delta\epsilon = 5\%$:

$$E_i = \frac{\sigma_{i,iniziale} - \sigma_{i-1,finale}}{\Delta\epsilon}. \tag{2.17}$$

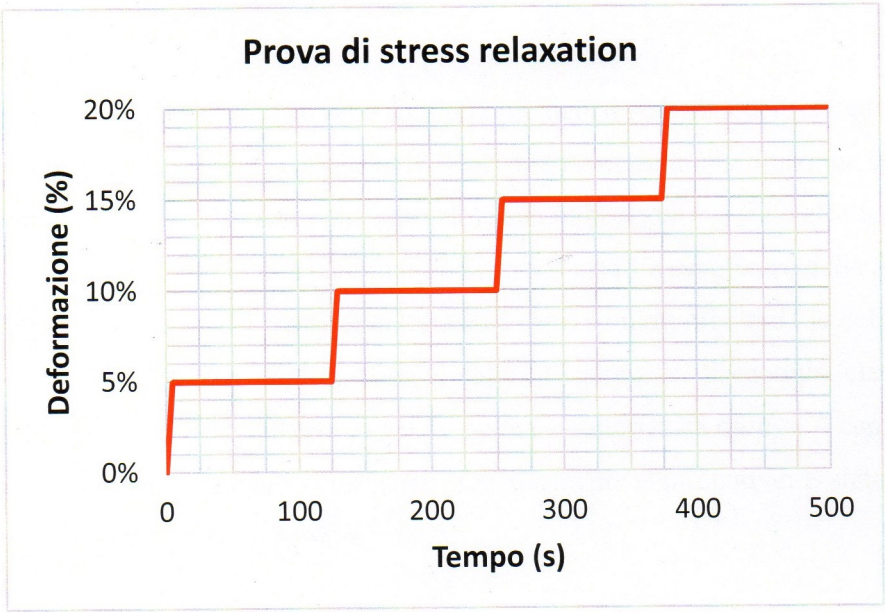


Figure 2.17: *Stress and relaxation test: ramps and deformations applied to the samples [36].*

For the first deformation ($\epsilon = 5\%$), the elastic modulus is calculated as $E_{5\%} = \frac{\sigma_{5\%,iniziale}}{0.05}$.

Finally it was also calculated the relaxation modulus as the difference between final stress of each deformation interval and the final stress of the previous interval, divided for the incremental deformation $\Delta\epsilon = 5\%$:

$$E_{R,i} = \frac{\sigma_{i,finale} - \sigma_{i-1,finale}}{\Delta\epsilon}. \tag{2.18}$$

For the first deformation ($\epsilon = 5\%$), the relaxation modulus is calculated as $E_{R,5\%} = \frac{\sigma_{5\%,finale}}{0.05}$.

2.7 Design and fabrication of the scaffold mold cap

It was designed a cap for the scaffold mold in order to prevent the contamination from bacteria present in the air. It was not possible to project a cap with Petri's

dishes design because silicone is a soft material whereas in Petri's design it is necessary to have hard materials, thus it was designed a cap which completely seals the scaffold mold except for a small opening which is mechanically fitted with an SFCA (Surfactant-Free Cellulose Acetate) filter. This filter (Figure 2.18) is normally used for aqueous solutions filtration but it was used to have a minimum gas exchange between the internal environment of scaffold mold which will contain a vascularized scaffold with embedded cells and the external environment of an incubator. The selected unit has a pore diameter of $0.2\mu m$ and a membrane diameter of $28mm$, these pores dimensions allow to prevent the contamination from the bacteria eventually present in the incubator because the average dimensions of air bacteria is major than $0.2\mu m$ [43]. Furthermore, in the cap, were designed four through holes where four screws (M4 ISO) can pass to fix it on a designed base, in order to seal the scaffold mold.

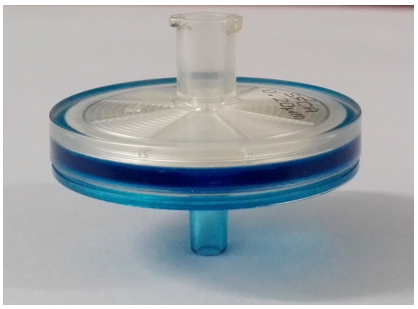
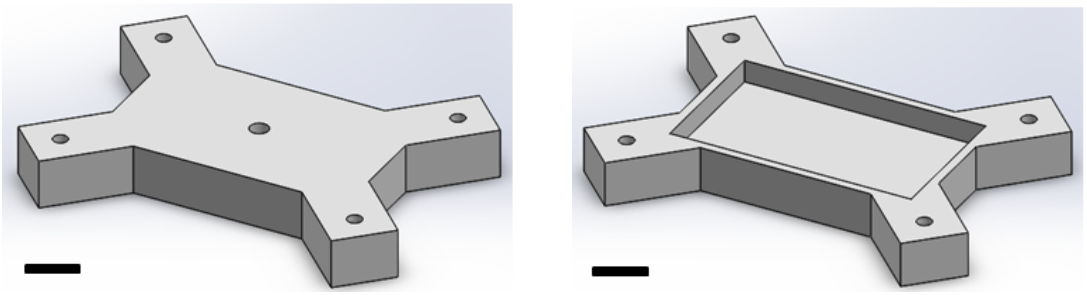


Figure 2.18: *Syringe filter Minisart ® NML.*

The base was designed to contain the scaffold mold and four threaded pores were obtained in it through a post processing, in order to fix the cap on it through the tighten of four screws.

The cap (Figure 2.19a) was printed with the 3D printer present in LaBS using a filament of PLA and the technique of Fused Deposition Modeling (FDM), then it was performed a post processing with a drill in order to: better define the through holes diameter where the four screw enter and the pore in which is mechanically fitted the filter.



(a) ***(b)***

Figure 2.19: *In the figure it is possible to see the drawings of: scaffold mold cap (a) and scaffold mold base (b) which were printed through the 3D FDM printer present in LaBS (scale bar: 20 mm).*

The base (Figure 2.19b), like cap, was printed through the 3D printer present in LaBS and in the post processing, with a drill, were created the four threaded holes where screws are tighten.

In the Figure 2.20 it is possible to see the entire assembled structure.

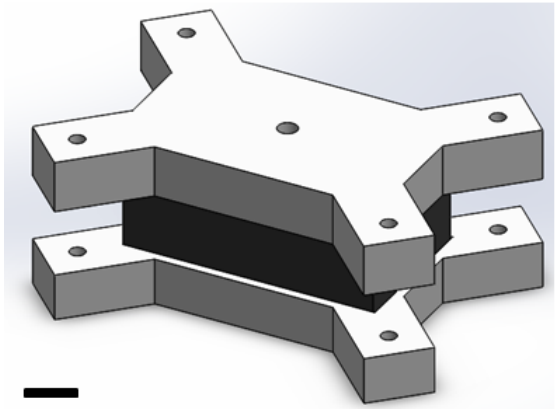


Figure 2.20: *Assembly of: cap (white), scaffold mold (black) and base (white). (Scale bar: 20 mm).*

Chapter 3

Results and discussion

3.1 Results of fluid dynamic simulations

Due to the results obtained by the simulations performed with Ansys Fluent, the peristaltic pump flow rate which perfuse the vascular network obtained within the hydrogel, is set at: $0.8\frac{ml}{min}$. This flow rate allow to obtain in all the central zone channels a velocity within the physiological range of values previously seen (between $0,1\frac{mm}{s}$ and $0,5\frac{mm}{s}$), with the exception of only the central vessel in which the velocity is slightly major than these values (Table 3.1) but considering that also they are approximation it can be evaluated as a positive result.
The simulation of mesh 3 allows to obtain the mass flow rate for all the outlets chosen which correspond to the central zone channels, dividing these values for the density of water ($1000\frac{Kg}{m^3}$) to find their flow rate and then using this formula:

$$Q = v * A \tag{3.1}$$

which is the inverse formula of equation 2.13 shown in Chapter 2, it is possible to calculate their velocities which are reported in Table 3.1. It is worth to notice that as it is possible to see in Table 3.1 the settings of gravity almost does not influence the difference in velocities between superior plane channels and inferior plane channels, thus allow to consider gravity negligible also in further studies with different capillaries networks due to its low influence when considering small dimensions.

Mass flow rate (Kg/s)	**Velocity (mm/s)**
$\dot{m}_1 = 6,74 * 10^{-7}$	$v_1 = 0,21$
$\dot{m}_2 = 1,12 * 10^{-6}$	$v_2 = 0,35$
$\dot{m}_3 = 1,12 * 10^{-6}$	$v_3 = 0,35$
$\dot{m}_4 = 6,74 * 10^{-7}$	$v_4 = 0,21$
$\dot{m}_5 = 1,67 * 10^{-6}$	$v_5 = 0,53$
$\dot{m}_6 = 2,17 * 10^{-6}$	$v_6 = 0,69$
$\dot{m}_7 = 1,67 * 10^{-6}$	$v_7 = 0,53$
$\dot{m}_8 = 1,64 * 10^{-7}$	$v_8 = 0,21$
$\dot{m}_9 = 1,12 * 10^{-6}$	$v_9 = 0,35$
$\dot{m}_{10} = 1,13 * 10^{-6}$	$v_{10} = 0,35$
$\dot{m}_{11} = 6,75 * 10^{-7}$	$v_{11} = 0,21$

Table 3.1: *Values of mass flow rate obtained from the simulation of mesh 3 performed with Ansys Fluent and velocities obtained through analytic calculations for all the pressure outlet chosen, which correspond to the central zone channels of the vascular network obtained within the hydrogel, numerated from left to right starting from the superior plane.*

The wall shear stress present in half of the designed vascular network which was utilized to perform the simulations, is resulted widely below the threshold of $0.5Pa$ (imposed considering a safety coefficient of 2) as it is possible to see in the Figure 3.1 extracted from the simulation performed with Ansys Fluent.

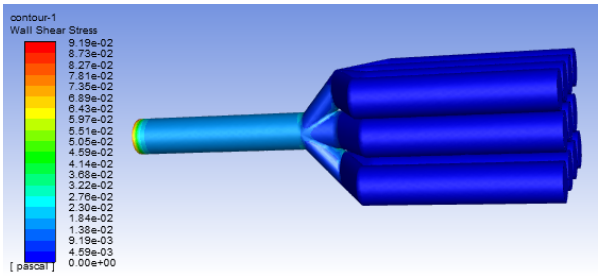


Figure 3.1: *Map of the wall shear stress present in half of the designed vascular network which was utilized to perform the simulations with Ansys Fluent.*

In the other half of the vascular network (not simulated with Ansys Fluent) are expected the same wall shear stress values due to the symmetry.

3.2 Results of hydrogel mechanical characterization

In the Tables 3.2 and 3.3 are reported the initial and final stresses respectively calculated with the force values obtained from the test machine.

Deformation	Sample 1	Sample 2	Sample 3	Sample 4	M	σ
5%	0.286	0.239	0.442	0.175	0.286	0.114
10%	0.605	0.490	0.831	0.344	0.567	0.206
15%	1.01	0.859	1.23	0.627	0.933	0.255
20%	1.58	1.40	1.76	1.06	1.45	0.297

Table 3.2: *Initial stresses measured in kPa for the 4 samples of hydrogel (alginate 50% - gelatin 50%) with their medium (M) and standard deviation (σ).*

Deformation	Sample 1	Sample 2	Sample 3	Sample 4	M	σ
5%	0.210	0.156	0.267	0.102	0.184	0.0711
10%	0.484	0.366	0.611	0.239	0.425	0.159
15%	0.834	0.662	0.815	0.468	0.695	0.170
20%	1.15	1.07	1.33	0.793	1.08	0.222

Table 3.3: *Final stresses measured in kPa for the 4 samples of hydrogel (alginate 50% - gelatin 50%) with their medium (M) and standard deviation (σ).*

The elastic and relaxation modulus are reported respectively in Tables 3.4 and 3.5, they are calculated through the equation 2.17 and 2.18 respectively.
Considering that the elastic modulus obtained for hydrogel composed by 50% of gelatin and 50% of alginate is approximately between 6 and $15kPa$, it can be used for application which regards soft tissues and in particular related to: skin,

Deformation	Sample 1	Sample 2	Sample 3	Sample 4	M	σ
5%	5.73	8.85	3.50	4.77	5.71	2.28
10%	7.89	11.3	4.84	6.68	7.67	2.71
15%	10.6	12.4	7.77	9.87	10.1	1.92
20%	14.9	18.8	11.8	14.8	15.1	2.87

Table 3.4: *Elastic modulus measured in kPa for the 4 samples of hydrogel (alginate 50% - gelatin 50%) with their medium (M) and standard deviation (σ).*

Deformation	Sample 1	Sample 2	Sample 3	Sample 4	M	σ
5%	4.20	2.04	5.35	3.12	3.68	1.42
10%	5.47	2.74	6.88	4.20	4.82	1.77
15%	7.00	4.58	4.07	5.92	5.40	1.32
20%	6.24	6.49	10.2	8.09	7.77	1.85

Table 3.5: *Relaxation modulus measured in kPa for the 4 samples of hydrogel (alginate 50% - gelatin 50%) with their medium (M) and standard deviation (σ).*

glands and muscles as can be seen in the Figure 3.2. Through the elastic modulus it is possible to define the tissues that can be obtained by culturing cells within the scaffold because, as previously said, the scaffold function is to support cells growth until the extra cellular matrix (ECM) produced is enough to support itself the cells, thus the elastic modulus of the scaffold must be similar to the tissue of application to provide an adequate mechanical support. Furthermore considering the possibility to use stem cells instead of differentiated cells, their differentiation

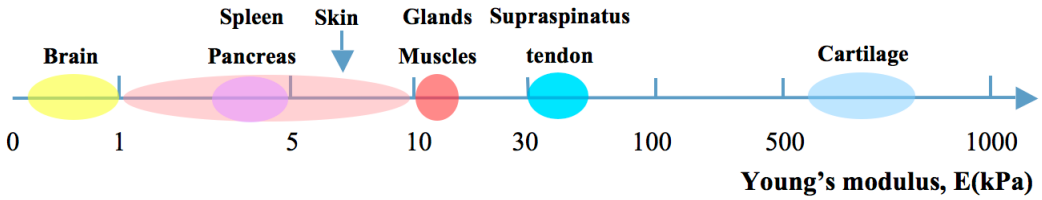


Figure 3.2: *Representation of human soft tissues elastic modulus [24].*

depends also from the elastic modulus of the tissue [24].

3.3 Vascular network printed and scaffold mold obtained

Through the 3D printer present in LaBS it was possible to print only one plane of the chosen vascular network, this is due to the fact that this 3D printer posses only one extruder. Thus, in the case of supports printed to obtain multiple planes, these are difficult to be removed manually when dealing with small dimensions (see Chapter 2).

Furthermore the vascular network dimensions were changed and the distance between the central zone channels was increased to $1.2mm$ instead of $0.2mm$ this is due to: a difficulty by the 3D printer (with a nozzle of $0.4mm$) to maintain this small distance between channels without fuse them together during printing and the increasing of channels dimensions when the vascular network is embedded into the hydrogel which is due to the absorption of water by PVA and can cause the channels conjunction if there is not a proper distance between them. Whereas the channels diameter chosen remains the same and it is equal to $2mm$ (Figure 3.3). The printing process to obtain a vascular network is not sterile, however this can be sterilized after the fabrication process for example through γ-rays which is a sterilization method compatible with thermo-sensitive materials.

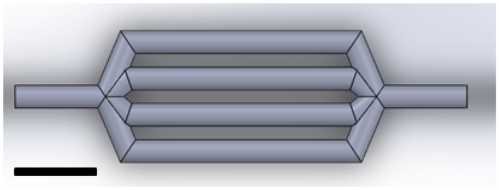


Figure 3.3: *In the figure it is possible to see the top view of the vascular network, which was designed and printed to be used to vascularize alginate/gelatin hydrogels using the procedure previously exposed in Chapter 2 (scale bar: 8 mm).*

The two pieces that compose the master which is utilized to produce a scaffold mold made by silicone rubber (Silplay 184/28), were printed in ABS through the 3D printer present in LaBS and combined to obtain the master (Figure 3.4). Acrylonitrile-butadiene-styrene (ABS) is a material which can release carcinogenic components at high temperatures but this can happen only during the printing process whereas, when the two pieces printed in ABS are combined to obtain the master and used for the production of silicone scaffold mold, there is no problem of carcinogenic components release and eventually the master or silicone scaffold mold obtained can be treated to remove them before their utilize. The pieces obtained in ABS have a visible retire of the material, but this is a typical disadvantage in the utilize of this material with 3D printing which can be overcome using a 3D printer with higher performances and maintaining warm the printed parts during all the printing process. However it was not used the PLA because, even if this material allow easily to obtain better printing than ABS, this last one is not degraded by water and has higher mechanical properties than PLA. It is also possible to use different fabrication processes and materials to obtain the scaffold mold, for example it is possible to use a Computer Numerical Control (CNC) machine to produce a scaffold mold with polymeric biomaterials such as: poly-(methyl methacrylate) (PMMA) or polycarbonate (PC).

The silicone scaffold mold formation was performed by mixing in a ratio 50:50 the components A and B, their reaction is a poly-addition reticulation which vulcanize at environmental temperature. The two components were mixed and poured into the master then it was placed in a freezer to remove the air bubbles eventually embedded during mixing procedure and after 10 hours it was removed from the freezer and left at environmental temperature for 24 hours allowing its solidification, finally the scaffold mold is removed manually from the master and this last one can be utilized again to obtain others scaffold molds with the same procedure. It is possible to observe that the air bubbles formation during the two components

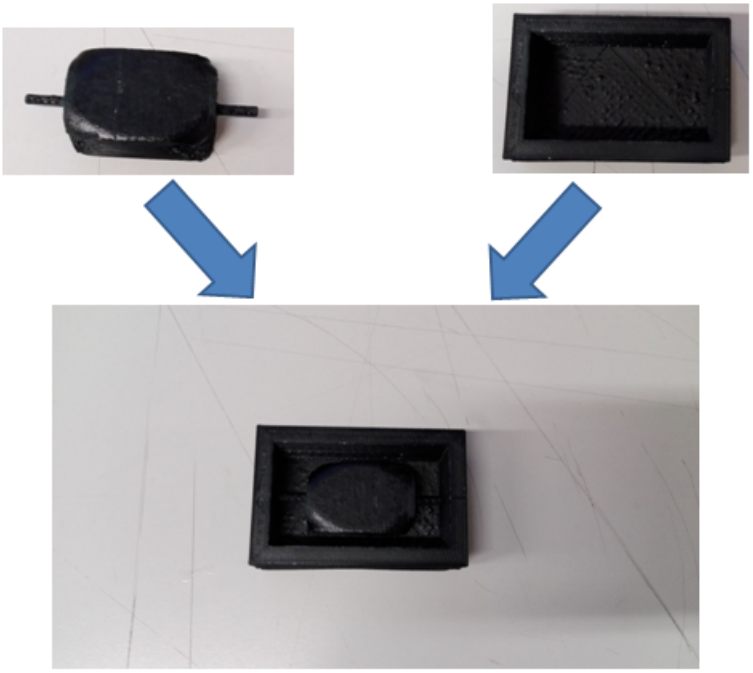


Figure 3.4: *In the figure it is possible to see the two pieces printed in ABS through the 3D printer and how they are combined to obtain the master needed to generate the scaffold mold in silicone.*

mixing is a relevant problem in scaffold mold production using this method because they can reduce significantly the mechanical resistance and moreover also the integrity of silicone scaffold mold. This silicon rubber (*Silplay 184/28*) can be sterilized and prepared to host a hydrogel solution containing cells, even if it is worth to notice that it is a food grade material, thus it will be better to substitute it with a biomaterial, for example poly-dimethylsiloxane (PDMS) is a biological grade silicone which can be utilized as scaffold mold.

The vascular network obtained is long enough to allow its suspension in the scaffold mold during the pouring of hydrogel solution, but it is not long as all the scaffold mold length, because the PVA dissolution is helped from the presence of water in the hydrogel whereas water is not present in the inlet/outlet silicone channels of the scaffold mold where the vascular network inlet/outlet are embedded and so cannot enhance PVA dissolution in these zones. The hydraulic connectors

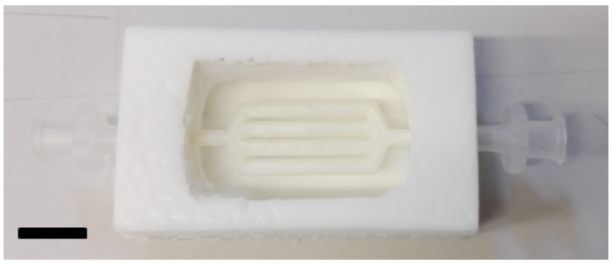
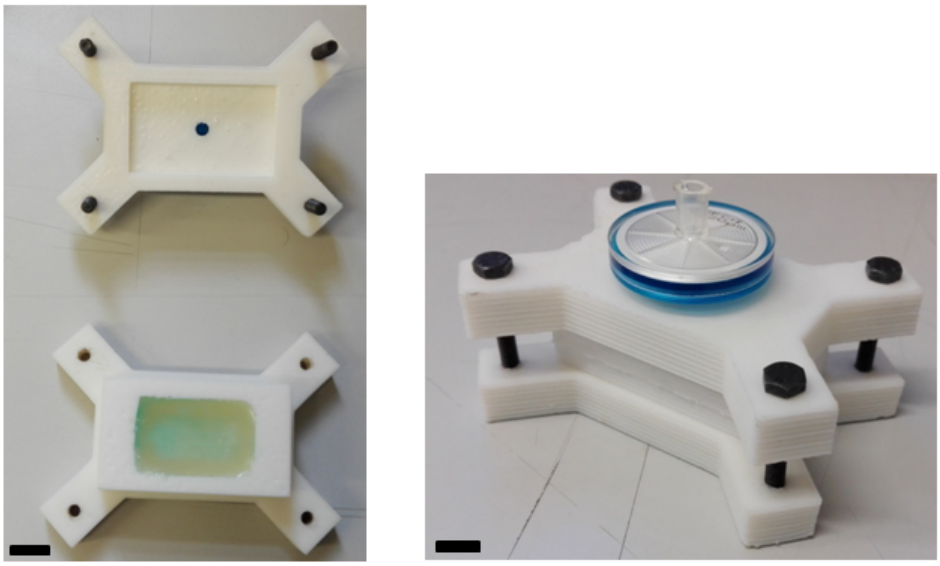


Figure 3.5: *In the figure it is possible to see the scaffold mold in silicone rubber (Silplay 184/28) obtained and the vascular network in PVA printed with the 3D printer FDM present in LaBS which is placed in it. Furthermore the hydraulic connectors are connected at the inlet/outlet of the scaffold mold (scale bar: 10 mm).*

occupy $5mm$ of the silicone inlet/outlet channels and are not in contact with the vascular network inlet/outlet, in this way they can be easily placed and removed even before the completely removal of PVA. In Figure 3.5 it is possible to see the silicone scaffold mold and how the vascular network is placed in it before the pouring of hydrogel solution.

3.4 Assembly of the device and vascular network removal

After the vascular network placement into the scaffold mold, it was prepared the hydrogel solution following the procedure exposed in Chapter 2 and focusing on the complete solubilization of calcium carbonate ($CaCO_3$) which is difficult because it has a low solubility in water but is important to free Ca^{2+} ions which cause the ionic gelation of alginate. The mixture of gelatin and alginate solutions was poured into the scaffold mold, this last one was placed on the base which has 4 threaded holes and the cap was sealed on it through 4 screws which enter in the cap through holes and are tighten to the base (Figure 3.6). The device obtained in this way was left in fridge at $4°c$ for one hour to allow the physical gelation of gelatin and simultaneously the ionic one of alginate.



(a) *(b)*

Figure 3.6: *In the picture it is possible to see the final device (base, scaffold mold containing a vascularized hydrogel and cap) open (a) and close (b). (Scale bar: 10 mm).*

The cap and the base were printed in PLA with the 3D printer present in LaBS and then post processed to: generate the threaded holes, improve the profile of through holes and the hole in which is inserted mechanically the filter. Polylactic acid is a biomaterial thus, it is compatible with cells but it is degraded by water; for this reason it is better to substitute it with another biomaterial which can withstand the environment of incubator which is full of moisture also for longer times, for example: polycarbonate (PC) or poly-methyl methacrylate (PMMA) which can be manufactured through a computer numerical control (CNC) machine.

The vascular network in PVA was removed, leaving channels in which a culture medium can flow, after at least 7 hours from gel preparation through the vascular network perfusion with a peristaltic pump (Mini Peristaltic Pump II, *Harvard Apparatus*) at the velocity of $0.8\frac{ml}{min}$. In Figure 3.7 it is possible to see the complete hydraulic circuit connected to the device. The complete hydrogel gelation happens in 1 hour but it is requested more time to remove the vascular network

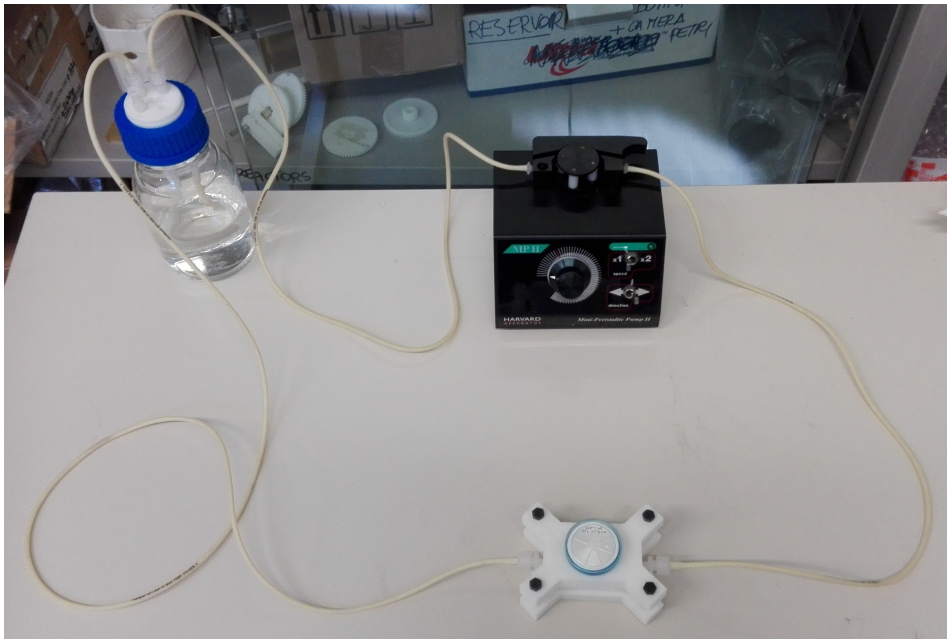


Figure 3.7: *The picture capture the hydraulic circuit, utilized to vascular network removal, connected to the device.*

through deionized water perfusion because the hydrogel mechanical characteristics are low, thus if the PVA vascular network do not dissolve easily the perfused water is absorbed by the hydrogel causing its swelling which can induce its destruction. Furthermore the hydrogel low thermal stability cause that it needs to be conserved in fridge at $4°c$, thus the water present in the hydrogel, which helps to dissolve PVA vascular network, is cold whereas this last one is dissolved better in water at high temperatures.

The time requested for vascular network perfusion is too much, thus in a case of cell-laden hydrogels, cells in time will probably find an environment which lack of nutrients and accumulate too much wastes, this situation would compromise their viability. In order to reduce the time requested for vascular network dissolution and its complete removal, it is possible to increment both the hydrogel mechanical and thermal stability. These hydrogel characteristics can be improved using a different kind of hydrogel, for example one composed only by gelatin which gela-

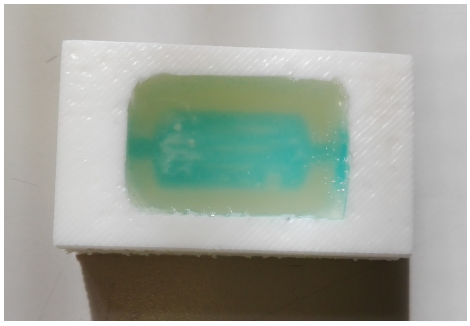


Figure 3.8: *The picture capture a vascularized scaffold in which the vascular network is visible due to the insertion of a blue water die in it.*

tion is not only thermal but also chemical through the utilize of transglutaminase (TG), like performed in their work by Rodrigo Pimentel C. et al. [17]; in this way the low thermal stability will be overcome and it will be possible leave the device in an incubator where cells find the environment adapt to their life and the temperature of $37°c$ help to dissolve PVA vascular network better than $4°c$. Also other kinds of hydrogel can be used, the important is that they will have an adequate mechanical and thermal stability to effectively allow cell culture in an incubator and PVA vascular network complete removal in times more compatible with the cells viability in hydrogels where they are embedded.

During the PVA vascular network removal it was utilized deionized water but this was possible only because there are no cells in the hydrogel, in the case of cell-laden hydrogels it will be necessary to use a physiologic solution or a culture medium to dissolve PVA because deionized water can cause cellular swelling and destruction due to osmosis. Furthermore with cell-laden hydrogels it will be also necessary to perform the vascular network removal in an incubator to maintain the conditions necessary to cell viability for the cells present into the hydrogel and after vascular network removal, seeding endothelial cells in the channel created into the hydrogel to mimic the natural blood vessels.

In order to see the vascular network obtained into the hydrogel it was utilized a blue water dye which was inserted from the scaffold mold inlet (Figure 3.8).

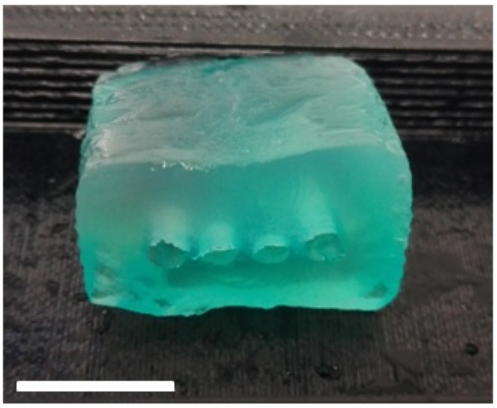


Figure 3.9: *In the picture it is possible to see the channels obtained into the central zone lateral section of a vascularized scaffold. The blue color is due to the blue water dye previously utilized to color the vascular network obtained into the hydrogel in order to see it (scale bar: 10 mm).*

In this way it was possible to observe that the vascular network was obtained, but with this method it was possible only to see it, thus through the utilize of calcium chloride ($CaCl_2$) on surface and through the vascular network obtained into the hydrogel, the alginate ionic gelation (due to Ca^{2+} ions) was improved and this allowed the hydrogel sectioning without its destruction and gave the possibility to measure the internal channels diameters. The precision of the measures is not elevate due to the fact that the material is soft and so during its sectioning the channels dimensions can variate. The dimensions of channels increased from $2mm$ to about $2.65mm$, this is due to the absorption of water by the PVA before its dissolution and must be considerate in the next vascular network designs to be obtained through this method using cell-laden hydrogels, because the risk is that the cells which are contained in the hydrogel, subjected to the stress caused by PVA swelling, can die and moreover that the channels can connect between them. These channels diameters are in the range of small arteries and not in the one of capillaries, but they can act as a path for the endothelial cells and then this last ones can generate new vessels with the dimensions of capillaries, furthermore using different 3D printing methods to obtain vascularized scaffold, previously seen

in the Chapter 1, it is also possible to obtain smaller diameters for the channels. In the Figure 3.9 it is possible to see the section of a vascularized hydrogel.
Even if the distance between channels was changed from $0.2mm$ to $1.2mm$ due to the difficulties to obtain the vascularized scaffold it must be evaluated the effective impact of it on the cells. In fact some authors observed, from practical evaluations of engineered vascularized construct, that cell viability is high even within 1 mm from the channels [44] [45].
The final dimensions for the vascular network printed to obtain the vascularized scaffold are: 40 mm of total length, 11.6 mm of total width.
The final dimensions of the obtained vascularized hydrogel are: 34x21.6x12.4 mm.
The obtained device is economic, in fact the cost of a 3D FDM printer with good performances is about 1500-2500 €, the PVA and ABS filaments cost about 30 € for 1 Kg of material, whereas the PVA filament costs about 35 € for 250 g of material. The 3D printer can be utilized several times to obtain a lot of prototypes and the filaments can be used a lot before they are completely consumed. The peristaltic pump utilized cost about 700 € but it can be used for others purposes and the reagents can be utilized several times, their cost is about: 40 € for alginic acid obtained from brown seaweed (A7003-100g, *Sigma Aldrich*), 55 € for gelatin obtained from bovine skin (Type B, G9391-100g, *Sigma Aldrich*), 55 € for sodium bicarbonate ($NaHCO_3$, S5761-500g, *Sigma Aldrich*), 35 € for sodium hydroxide ($NaOH$, 71692-1Kg, *Fluka Analytical*), 50 € for calcium carbonate ($CaCO_3$, C4830-100g, *Sigma Aldrich*) and 35 € for $D-(+)-glucono-\delta-lattone$ (GDL, G4750-100g, *Sigma Aldrich*). Thus, with a total of approximately 3600 € it is possible to obtain a lot of vascularized scaffold following this procedure, for this reason it is possible to define it economic.

Chapter 4

Conclusions and future developments

In the present thesis work it was designed a procedure and a vascular network to vascularize scaffolds in order to overcome the major limitation in tissue engineering: the lack of a vascular network in engineered tissues.
It was obtained a vascularized scaffold with a simplified version of the originally projected vascular network following the designed procedure and this demonstrate the effective applicability of this procedure, inspired by the one utilized in literature by Rodrigo Pimentel C. et al. [17].
The vascular network originally designed follows an engineering approach instead of a biomimetic one, thus the target was to project a vascular network which would supply the cells present in scaffold core with an adequate amount of nutrients and oxygen also neglecting biomimetic considerations such as Murray's law. This vascular network posses the innovative characteristic to be developed on multiple planes, but it can be obtained only with a double extruder 3D FDM printer, thus through the 3D FDM printer present in LaBS, which has only one extruder, it was possible to obtain only a single plane of this network.
The hydrogel utilized to obtain the scaffold posses the disadvantages to have low mechanical characteristics and low thermal stability and these increase the time requested for vascular network complete removal through active perfusion as pre-

viously reported, thus in future developments it will be necessary to change the composition of hydrogel or to modify its gelation mechanism in order to give him higher mechanical characteristics and thermal stability in order to reduce the time necessary to PVA vascular network removal to times compatible with cells viability. Furthermore with the same objective it must be necessary reduce the gelation time from 1 hour to few minutes.

The channels diameters obtained are in the range of small arteries, thus it is necessary to reduce their dimensions and this can be done, following the same procedure, through the utilize of 3D FDM printers with higher performances to print the vascular network in PVA. Furthermore it is evident from the present thesis work and the precedent work performed by Rodrigo Pimentel C. et al. [17] that the PVA increases its dimensions before being removed due to water absorbency, thus it must be necessary to consider this phenomenon during the design of vascular network to vascularize scaffold using this procedure and try to reduce it for example by reducing the time necessary to remove PVA vascular network through active perfusion.

For the originally designed vascular network it was also performed a computational fluid dynamic analysis with the objective to define the flow rate through which it is possible to perfuse the vascularized scaffold with a culture medium to prevent cell detachment caused by an elevated shear stress and to obtain a physiological velocity in the central zone of the vascular network. From this analysis resulted very low values of velocity which will be reduced again if the diameters of channels printed will be lower, thus it must be considered also the utilize of micro-pumps in future developments.

From the mechanical characterization it was emerged that the hydrogel used in the present thesis work can be utilized as a scaffold for: muscular tissues, skin and glands. However, hydrogels can be tuned to reach different elastic modulus, furthermore in the case their mechanical characteristics increasing, as previously

said to help the vascular network removal, it must be necessary to perform other mechanical characterizations to identify the correct tissue for which a chosen hydrogel can be used as scaffolds.

This work represent a forward step in the scaffold vascularization because it confirms the possibility to use an economic procedure recently developed to vascularize scaffolds. In the successive studies it must be necessary try to obtain the complete vascular network into the same or better hydrogel to confirm the versatility of the procedure, which can be applied to different kind of vascular networks, thus also to biomimetic ones and, once it is obtained, perform the same procedure with cells to collect data on the real possibility to obtain vascularized engineered constructs through this procedure.

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