

École polytechnique de Louvain

Pore size profile and thermal degradation of collagen-based porous material used in hemostasis

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ABSTRACT

HemopatchTM Sealing Hemostat is a NHS-PEG-coated collagen pad and hemostatic agent designed to help stop or limit bleeding during surgery. It is a Baxter product, available on the market, yet no information regarding its manufacturing or structure are made available by the external manufacturers. This thesis presents a quantified characterization of the collagen pad's porosity, as well as an investigation into the pad's thermal degradation.

The collagen scaffold proved to be a highly porous material, with an estimated relative density between 1 and 3 %. It is quite isotropic, but presents a slight fiber preferential direction in the plane parallel to the pad's surface. Most pores have a diameter superior to 10 μm and present a low interconnectivity.

When heated, the collagen pad denatures at $\sim 75\text{ }^{\circ}\text{C}$, and, above $200\text{ }^{\circ}\text{C}$, it shrinks isotropically. When it has reached a maximum shrinking, its relative density has increased to $\sim 46\text{ }%$, which makes it stiffer by almost three orders of magnitude. The pore size also decreases but most pores retain a diameter superior to 5 μm . Finally, by heating the pad with a pre-heated grid, this thesis also demonstrates the possibility to degrade the collagen pad only partially. This offers a possibility to adjust the pad's structure after its manufacturing in order to improve its hemostatic properties, which could further be explored.

Contents	v
Abbreviations and symbols	vii
List of Figures	ix
List of Tables	xi
Introduction	1
1 State of The Art	3
1.1 Collagen: structure, functionalities and applications	3
1.1.1 Structure and composition	3
1.1.2 Functionalities and applications	5
1.2 Surgical sealants and hemostatic agents	9
1.2.1 Types of sealants and hemostatic agents	10
1.3 Porosity characterization	13
1.4 Thermal degradation	17
1.4.1 Collagen degradation and denaturation	17
1.4.2 Thermal degradation characterization methods	19
2 Materials & Methods	21
2.1 Materials	21
2.1.1 Collagen-based porous material	21
2.1.2 Chemicals	21
2.2 Visualization techniques	22
2.2.1 Optical microscopy	22
2.2.2 Scanning electron microscopy	22
2.2.3 Confocal microscopy	23
2.2.4 X-ray micro-computed tomography (Micro-CT)	24
2.3 Relative density measurement	24

CONTENTS

2.4	Particle size analysis	25
2.4.1	Occhio	25
2.4.2	Parenteral Particle Counter	26
2.5	Thermal analysis techniques	28
2.5.1	<i>In situ</i> heating	28
2.5.2	Visualization of the change in porosity due to heating	29
2.5.3	Differential scanning calorimetry (DSC)	29
2.5.4	Dynamic mechanical analysis (DMA)	30
2.5.5	Thermomechanical analysis (TMA)	30
2.5.6	Thermogravimetric analysis (TGA)	31
2.5.7	Chemical degradation with temperature	31
3	Results & Discussion	33
3.1	Scaffold visualization	33
3.1.1	Surface	33
3.1.2	Cross-sections	34
3.1.3	3D-visualization	36
3.2	Porosity quantification	39
3.2.1	Relative density	39
3.2.2	Pore size distribution and fiber thickness distribution	41
3.2.3	Pore size distribution and pore interconnectivity of pad as humid membrane	42
3.2.4	Degree of anisotropy	46
3.3	Thermal degradation	50
3.3.1	Shrinking of the pad	50
3.3.2	Relative density after degradation	54
3.3.3	Pore size distribution and pore interconnectivity of degraded pad as humid membrane	55
3.3.4	Possibility to partially degrade the pad	57
3.3.5	Thermal transitions identification through DSC	58
3.3.6	Thermo-mechanical behavior evolution of the pad	62
3.3.7	Change in pad thickness with temperature	65
3.3.8	Mass loss evolution with temperature	66
3.3.9	Thermal evolution of molecular structure by FT-IR spectroscopy	67
	Conclusion	73
	Bibliography	75

ABBREVIATIONS AND SYMBOLS

Symbol	Description	Unit
AFM	Atomic force microscope or microscopy	
CTAn	Skyscan CT-analyser, Bruker micro-CT software	
CTVox	Skyscan 3D-rendering and visualization Bruker micro-CT software	
DA	Degree of anisotropy	
DMA	Dynamic mechanical analysis	
DSC	Differential scanning calorimetry	
E	Stiffness	MPa
ECM	Extracellular matrix	
FPS	Frames per seconds	s ⁻¹
FT-IR	Fourier-transform infrared	
G'	Storage modulus	MPa
G''	Loss modulus	MPa
Gly	Glycine	
GRF	Gelatin-resorcinol-formaldehyde	
H-bond	Hydrogen bond	
HIT	Hydrothermal isometric tension	
Hyp	Hydroxyproline	
LED	Light-emitting diode	
Micro-CT	X-ray micro-computed tomography	
NHS	N-hydroxysuccinimide	
PAH	Poly(allylamine hydrochloride)	
PBS	Phosphate-buffered saline	
PEG	Polyethylene glycol	
PIDS	Polarisation intensity differential scattering	
PMS	Particle Measuring Systems®	
PPC	Parenteral Particle Counter	
Pro	Proline	
PS	Polystyrene	
ρ^*	Scaffold density	g/cm ³

Symbol	Description	Unit
ρ_s	Collagen dry density	g/cm^3
MIL	Mean intercept length	
SEM	Scanning electron microscope or microscopy	
SNR	Signal-to-noise ratio	
SSA	Specific surface area	μm^{-1}
$\tan \delta$	Damping, ratio of storage over loss modulus	
TGA	Thermogravimetric analysis	
TMA	Thermomechanical analysis	
$\varnothing$	Collagen fiber outer diameter	μm

LIST OF FIGURES

1.1	The structural hierarchy of fibrillar collagen	4
1.2	Collagen triple helix featuring the telopeptides and pro-peptides . . .	4
1.3	Collagen-based materials used in biomedicine	6
1.4	Freeze-drying process for collagen scaffolds fabrication	7
1.5	Major components and steps of hemostasis	9
1.6	Hemopatch TM , collagen pad designed for hemostasis and sealing . . .	12
1.7	Typical SEM image of a collagen scaffold showing its “cellular” structure	14
1.8	Tetrakaidecahedron model used to quantify pore geometry of low-density porous materials	14
1.9	Effect of the heating rate on the denaturation endotherm of a glutaraldehyde-fixed porcine pericardium measured with DSC	20
2.1	Occhio Flow Cell Device	26
2.2	Set-up for filtration of suspension through collagen pad	27
2.3	Heating stage connected to SEM used for <i>in situ</i> heating of collagen pad	28
2.4	Collagen sample with 30- μ m-diameter particles in every other square of the grid	29
2.5	Schematic representation of DMA shearing deformation mode	30
3.1	Gold-coated collagen scaffold imaged with SEM	34
3.2	Gold-coated collagen scaffold imaged with SEM showing collagen D-period	34
3.3	Gold-coated collagen scaffold cross-sections imaged with SEM	35
3.4	Collagen scaffold imaged with confocal microscopy	36
3.5	3D-reconstruction of the collagen pad obtained with micro-CT	37
3.6	Structure separation and thickness distribution as measured with micro-CT 3D-analysis	41
3.7	Particle size distribution obtained with PPC before and after filtration through the collagen pad	43
3.8	Particle size distribution obtained with Occhio for suspensions with standard size particles	45

LIST OF FIGURES

3.9	Particles detected by Occhio in the suspension filtered through the collagen pad, displaying residues among the standard size particles . .	46
3.10	Degree of anisotropy (DA) calculation elements	47
3.11	Spherical-coordinate system used as reference to analyze the fibers' preferential directions	48
3.12	θ -orientation distribution of the collagen fibers	48
3.13	ϕ -orientation distribution of the collagen fibers	49
3.14	textitIn situ heating of the collagen pad imaged with optical microscope	50
3.15	Undegraded collagen sample, degraded samples after heating at 200 °C for 20 minutes and after heating at 225 °C for 4 minutes in an oven	52
3.16	Collagen pad unmodified (left), heated at 200 °C for 20 minutes (middle) and heated at 225 °C for 4 minutes (right), imaged with SEM	52
3.17	Distance measurements between standard size particles on the collagen pad for determining whether the shrinking of the pad occurs isotropically	53
3.18	Horizontal and vertical tracking of distances on the collagen pad with SEM as a function of temperature, showing the shrinking occurs isotropically	54
3.19	Particle size distribution obtained with PPC after filtration through unmodified and shrunk collagen pads	56
3.20	Collagen scaffolds imaged with SEM, showing a few pore diameter measures	56
3.21	Collagen pad partially degraded using a pre-heated grid	58
3.22	Heat flow as a function of temperature of unmodified and modified collagen pads, measured with DSC, with a heating rate of 5 °C/min .	59
3.23	Heat flow as a function of temperature of collagen pad, with alternating heating and cooling ramps (5 °C/min), measured with DSC	60
3.24	Heat flow as a function of temperature of degraded pad beforehand, with alternating heating and cooling ramps (5 °C/min), measured with DSC	61
3.25	Evolution of the storage modulus G' , loss modulus G'' and damping $\tan \delta$ of Hemopatch TM 's collagen pad with temperature	63
3.26	Evolution of G' , G'' and $\tan \delta$ with temperature of Duraform [®] Dural Graft Implant's collagen and of Hemopatch TM 's collagen	64
3.27	Evolution of the relative thickness of two collagen pads with temperature measured with TMA	65
3.28	Mass evolution of Hemopatch TM 's collagen pad with temperature, measured with TGA	66
3.29	FT-IR spectra of collagen pad between 25 °C and 225 °C	67
3.30	Region of FT-IR spectrum of collagen pad at 25, 75 and 175 °C . . .	68
3.31	Region of FT-IR spectrum of collagen pad between 25 °C and 175 °C	69
3.32	FT-IR spectrum with elements for calculation of intensity ratio $\frac{I_{1235}}{I_{1450}}$.	70

LIST OF TABLES

2.1	Samples prepared for confocal microscopy	23
2.2	Concentration of the suspensions containing standard size particles for analysis with Occhio	26
2.3	Concentration of the suspension containing standard size particles for analysis with Parenteral Particle Counter (PPC)	27
3.1	Synoptic table of visualization methods	38
3.2	Different relative density values from literature on other collagen scaffolds and computed in this thesis on Hemopatch TM 's collagen scaffold	40
3.3	Structure thickness distribution obtained with micro-CT 3D-analysis	42
3.4	Dimensions of the unmodified sample, a degraded sample after heating at 200 °C for 20 minutes and a degraded sample after heating at 225 °C for 4 minutes in an oven	52
3.5	Comparative table of the relative density of the unmodified and ther- mally degraded collagen samples	54
3.6	FT-IR bands intensity ratio $\frac{I_{1235}}{I_{1450}}$	70

Collagen-based porous materials are three-dimensional materials used in various clinical and medical applications, owing to collagen's unique supramolecular structure, its natural prevalence in human and animal tissues, the matrix it can provide for cell growth, as well as its biocompatible and biodegradable profile. Among the various fields of application of collagen materials, hemostasis is one of them. It refers to the physiological process occurring in the body during a blood loss, allowing the stop or reduction of the bleeding. Hemostatic patches composed of collagen scaffolds are one type of hemostatic or sealing agent being researched and adopted. Their purpose is to mimic and amplify the body's inherent hemostatic mechanisms.

HemopatchTM Sealing Hemostat is a collagen-based pad, offering hemostatic (capable of inducing coagulation) and sealing (providing a liquid-tight barrier) properties, used by surgeons to control bleeding during surgery. [1] The collagen pad is covered on one side with a thin layer of NHS-PEG¹, which favors tissue adhesion. It is a Baxter product and distributed by Baxter thanks to the support of different external manufacturers.² Despite its availability on the market since 2013, no data regarding the collagen pad's fabrication process or thorough characterization are available due to the intellectual properties kept by the subcontractors of HemopatchTM.

In this context, the first objective of this thesis aims at providing a characterization of the collagen pad's structure. As it is a highly porous material, characterizing the pad's porosity is essential not only to better understand its behavior *in vivo*, but also to further optimize it. A quantitative characterization of the pad's porous architecture might in turn provide an insight into some parameters which could be adjusted to change the pad's properties.

One way in which the structure of collagen scaffolds can be altered is through thermal treatment. In fact, it is known that collagen shrinks upon heating. Therefore, in the frame of this project and as a coherent continuity of the thesis' first objective,

¹NHS-PEG is a polymer which stands for N-hydroxylsuccinimide (NHS) functionalized polyethylene glycol (PEG).

²Baxter is an international healthcare company.

the second part explores the pad's structural alterations with temperature. This investigation was done in view of potentially designing a pad with various pore sizes in different locations or with a heat-controlled pore-size gradient, for instance.

In order to integrate both the characterization of the pad's porosity as well as its thermal degradation analysis, this thesis has been organized as follows.

Chapter 1 provides a description of the composition and structure of collagen, as well as an overview of some of its medical applications. Then, an outline of various hemostatic agents is presented, collagen and non-collagen based, among which HemopatchTM. Different ways of characterizing collagen scaffolds' porosity found in literature are introduced. Finally, the current state of the art with regards to collagen thermal degradation - the process and various analytical techniques - are discussed.

Chapter 2 covers the methods used both for the pad's porosity characterization and for the thermal degradation analysis.

Finally, Chapter 3 presents the results acquired along this thesis and discusses them. First, various visualizations of the collagen scaffold are shown. Thereafter, the scaffold's porosity is quantified with different parameters. Ultimately, the results of its thermal degradation investigation are presented and discussed.

1.1 Collagen: structure, functionalities and applications

1.1.1 Structure and composition

Collagen is a fibrous protein found in the extracellular matrix of many connective tissues in mammals and provides structure, rigidity and mechanical resilience to tissues. [2] It makes up about one third of total body protein in humans and most higher vertebrates. Collagen is organized in a structural hierarchy, which allows it to provide stiffness and strength to tissues. Its basic unit is the collagen triple helix or “tropocollagen”. The triple helix is considered as the tertiary structure of collagen. Each tropocollagen molecule is constituted of three left-handed polypeptide alpha helices, also known as α -chains, of identical length. They form the secondary structure of the protein. Three α -chains are joined together to form a single right-handed spiral of approximately 1.5 nm in diameter and 280 nm in length (lower part of Figure 1.1). [3]

Additionally, each tropocollagen molecule has at its ends short nonhelical domains, which are referred to as telopeptides (not displayed in Figure 1.1 but in Figure 1.2). They play a crucial role in the covalent crosslinking of the α -chains and they add flexibility to the molecule. [3]

The sequence of amino acids in collagen contains large numbers of glycine (Gly), proline (Pro) and hydroxyproline (Hyp). Glycine allows hydrogen-bonding between adjacent chains which holds together the three collagen subunits into a closed packed triple helix. Hydroxyprolines also form hydrogen bonds and help to stabilize the triple helix. On the other hand, the tropocollagen molecules are covalently crosslinked to each other to form long strands.

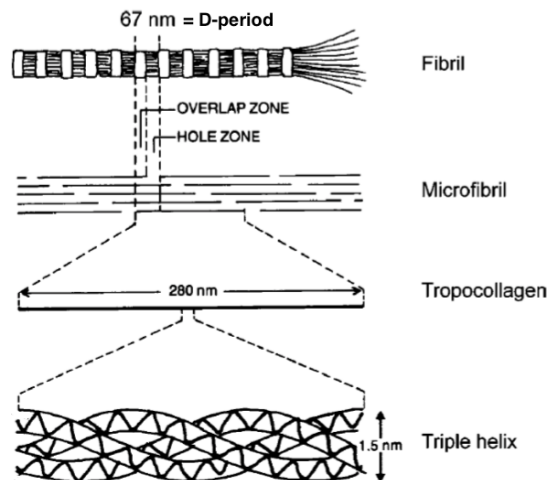


Figure 1.1: The structural hierarchy of fibrillar collagen [4]

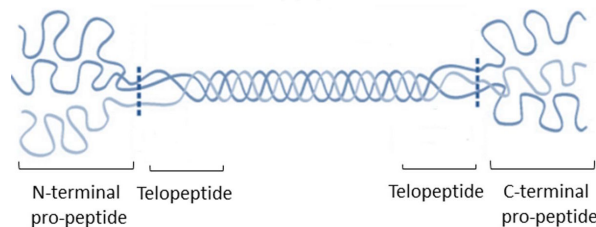


Figure 1.2: Collagen triple helix featuring the telopeptides and pro-peptides [5]

Five tropocollagens aggregate to form microfibrils; this is why the structure of collagen is considered supramolecular, providing collagen with a unique quaternary structure. Neighboring molecules pack together at about one quarter of their length or every 67 nm, which is called the D-period. This is why the microfibrils are much longer than tropocollagen molecules.

Microfibrils group to create fibrils which are 50-500 nm in diameter (middle and upper parts of Figure 1.1). Finally, fibers, constituted of fibrils, have a diameter between 50 and 300 μm . [4] [6]

The hierarchical packing of collagen molecules as described here above provides structural stability, mechanical integrity and enzymatic resilience to collagen-based tissues. [3] For instance, the triple helix is resistant to cleavage by enzymes such as pepsin and trypsin, as long as the triple helix is intact and tightly folded. [3] [7]

Collagen types Collagen comes in all shapes and sizes. The hallmark of a collagen is a molecule that is composed of three polypeptide chains forming a triple-helical motif as described here above, each of which contains regions characterized by the repeating amino acid motif (Gly-X-Y), where X and Y can be any amino acid, but are often occupied by proline (28 %) and hydroxyproline (38 %), as these strongly contribute to stabilizing the triple helix. [5] [8]

In humans, there are currently 28 different proteins known as collagen, and they are differentiated by a type number, from I to XXVIII. They can be distinguished by

their differences in the α -chains, among other criteria. [7] They can be recognized through their characteristic (Gly-X-Y) sequences and their homologies with other collagens. [3] Here under is an overview of several of the most common types and where they are naturally found in the body:

- Type I is the most widely-occurring collagen found in skin, ligament, tendon and bone.
- Type II is the primary collagen in cartilage.
- Type III is found in quite elastic tissues such as embryonic skin or blood vessels.
- Type IV is a network-forming collagen which forms X-shaped complexes and is abundant in basement membranes. It is the most known non-fibrillar collagen, i.e. it does not form large fibril bundles as shown in Figure 1.1, but a two-dimensional reticulum, and the molecules are longer than in fibrillar collagens. [4]
- Type V is found in minor amounts in association with type I collagen and is mostly found in the cornea. [9]

Types I, II and III make up 80 to 90 % of the total human body's collagen. [3] The collagen pad characterized in this thesis is composed of types I, III and V, which are all fibrillar collagens.

1.1.2 Functionalities and applications

Collagen is important for a broad range of functions such as tissue scaffolding, cell adhesion, cell migration and tissue repair. [7] It has found many applications in food, cosmetic, pharmaceutical and biomedical fields. In fact, collagen's natural prevalence in human tissue, as well as its wide array of convenient properties - such as its ability to form 3D scaffolds, its cytocompatibility and biodegradability - make it a frequent choice as raw material to manufacture cosmetic products and tissue-engineered scaffolds for various clinical applications. Furthermore, because it is already a constituent of the body, it will not be perceived as foreign matter which makes it an attractive biomaterial. The collagen used for the pad characterized in this thesis is an animal-extracted material, derived from bovine epidermis, which can still provoke an immune response or lead to the transmission of disease, but this is out of the scope of this thesis. [3]

Collagen-based materials in biomedicine

Collagen is widely used to create biomedicine and biomedical materials in various forms. The idea is to try and mimic the complex native tissue structure, at different levels and to different extents. As described for each type of collagen-based material here below, collagen can show various adequate and useful properties and is therefore used for many biomedical applications. Figure 1.3 features seven examples of how collagen is used to create different devices used in biomedicine.

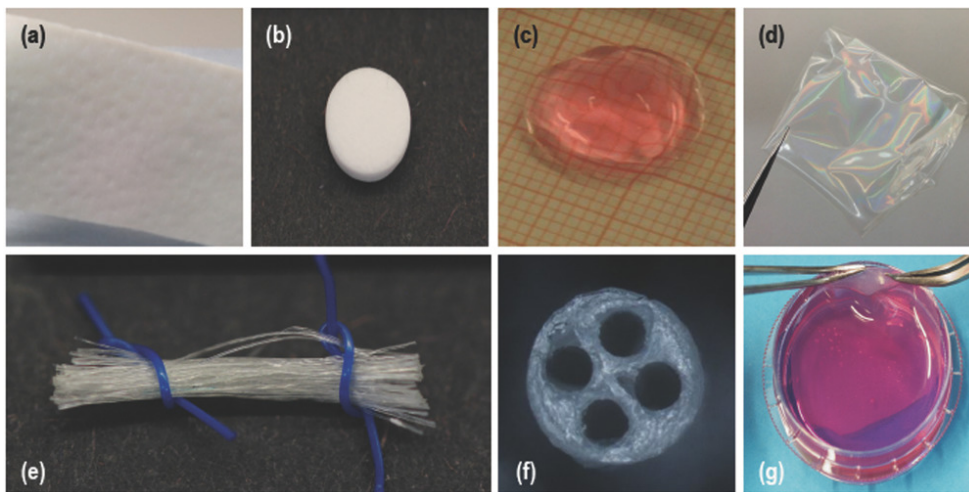


Figure 1.3: Collagen-based materials used in biomedicine. (a,b) Tissue grafts and sponges, also known as scaffolds (c) Hydrogels (d) Films and tubes (e) Self-assembled fibers (f) Multichannel neural conduits (g) Living tissue substitutes [3]

Collagen scaffolds As Lin *et al.* (2019) put into words, “scaffolds are sponges that are mainly composed of collagen with a porous 3D construction containing a larger length scale organization, i.e., architecture.” [10] The collagen material characterized in this thesis is for instance a collagen scaffold. Such materials (Figure 1.3 (a & b)) can be manufactured by precipitation, critical point drying, or freeze-drying. [11] It is known that the collagen pad characterized in this thesis has been made through freeze-drying, but the specific conditions and parameters of the process are kept protected by the supplier for intellectual property reasons. However, the general process of freeze-drying is described here below.

Freeze-drying (also known as ice-crystal templating or lyophilization) is a dehydration process used for the construction of highly porous implantable devices. [3] It is the primary manufacturing method of collagen scaffolds because it can almost maintain the native structural form and biological properties of both the collagen and the potential incorporated biomolecules. [10] [12]

The freeze-drying process is usually done in the following way. A collagen suspension is prepared, containing isolated fibrillar or microfibrillar collagen, water, and acetic acid or hydrogen chloride to adjust the pH value to around 3 (Figure 1.4, step 1). According to Varley *et al.* (2016), the suspension is maintained at 4 °C during the mixing process to prevent denaturation of the collagen fibers. [13] However, it appears that this procedure still leads to the partial denaturation of 3 - 6 % weight of the collagen. [11] The suspension is then freeze-dried by cooling to -40 °C at a rate of -0.9 °C/min (Figure 1.4, step 2) and held for 60 minutes. This creates a network with interpenetrating ice-crystals. [10] Then, the ice-crystals are sublimated under a vacuum of 50 mTorr (6.66 Pa) for 17 hours at 0 °C (step 3). Afterwards, the porous scaffold is obtained (step 4). [13] Finally, the scaffold can be crosslinked, either physically, chemically or biologically, depending on the application and the desired properties.

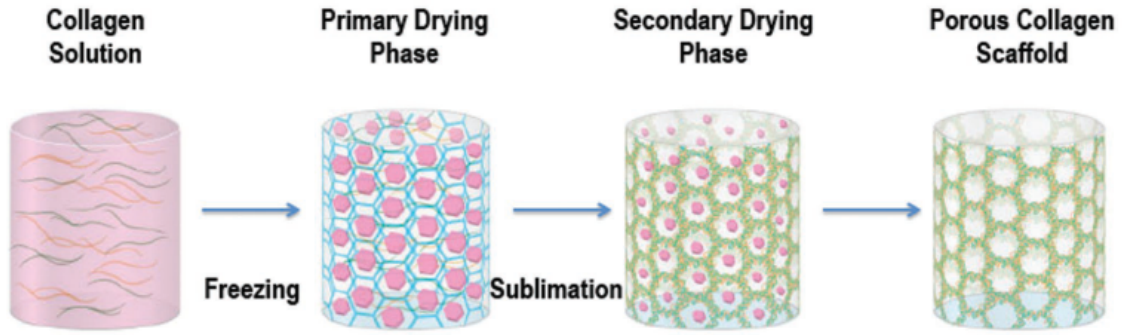


Figure 1.4: Freeze-drying process for collagen scaffolds fabrication [3]

The freezing rate (Figure 1.4, step 2) will have an effect on the size of the pores, the relative fraction of pores in the scaffold but also the homogeneity of the pores in the scaffold. For instance, the rate of $-0.9\text{ }^{\circ}\text{C}/\text{min}$ mentioned here above has been shown to provide the most homogeneous pore diameter i.e. with the smallest variance and the smallest pore diameter, compared to $-4.1\text{ }^{\circ}\text{C}/\text{min}$ (considered as quenching), $-0.7\text{ }^{\circ}\text{C}/\text{min}$ and $-0.6\text{ }^{\circ}\text{C}/\text{min}$. [12] Furthermore, not only does freezing rate have an impact on pore size, but the freezing temperature as well: freezing at relatively high temperatures will form large ice crystals and thus increase the pore size while freezing at low temperatures will have the opposite effect. [3] Pore size can also be controlled by using pre-prepared ice particulates as a porogen material. [14] The disadvantages of freeze-drying include long fabrication time as well as high energy consumption. [10]

Collagen scaffolds are used to enhance various types of cell cultures, to improve cell attachment and proliferation, to help bone regeneration or as hemostatic agent, as will be discussed in Section 1.2. These scaffolds can be loaded with cells such as stem cells, protein, or minerals such as hydroxyapatite which provides these scaffolds with an additional bioactivity both *in vitro* and *in vivo*.

In the end, no additional information is provided for the collagen pad characterized in this thesis. It is only known that the freeze-drying process somewhat physically crosslinks the collagen, and that the scaffold is not chemically crosslinked.

Hydrogels These materials are water-swollen, crosslinked networks which present properties similar to soft tissues, more than any other polymeric biomaterial. They entrap significant amounts of fluids, allowing the exchange of ions and nutrients with surrounding tissues. Collagen hydrogels (Figure 1.3 (c)) are used for tendon repair and regeneration, cartilage repair, as substrate for the growth of ocular-specific cell populations in the eye or even skin replacement. [3] The traditional fabrication process includes two steps. First, collagen monomers in acidic solutions aggregate to form fibrils at $4\text{ }^{\circ}\text{C}$ by the addition of NaOH and a phosphate buffer. Afterwards, the gelation is triggered by warming up the solution to physiological temperatures, thus in a self-assembly fashion. [10]

The main advantage of collagen hydrogels is that cells or other bioactive components, such as stem cells, drugs and growth factors, can directly be incorporated into the hydrogel during the fabrication process, by adding them to the collagen solution, in order to complete or improve tissue regeneration. [10]

Films and tubes Collagen films have been extensively used for cornea repair as they are transparent (Figure 1.3 (d)) and fulfill the mechanical requirements of the tissue. [15] In fact, they exhibit similar diffusion and mechanical properties as the stromal layer of the cornea. Collagen films are also used in the wound healing area as they support cell attachment, and encourage or maintain various types of cell growth. [3] They can once again be functionalized or crosslinked in various ways to yield specific biological properties. Collagen films wrapped in tubes have been used as nerve guidance, supporting axonal re-growth. [16] Figure 1.3 (f) shows a multichannel collagen neural conduit.

Self-assembled fibers Collagen fibers production methods offer the opportunity to tailor the mechanical properties of the fibers according to the target clinical application of interest. In fact, produced collagen fibers show structural and mechanical properties similar to native tissue and are used to improve bone and vascular cell differentiation, among others. [3] Figure 1.3 (e) shows a bundle of extruded collagen type I fibers. Just like for hydrogels, collagen fibers' bioactive properties can be enhanced with functionalization strategies with other protein such as decorin and resilin.

Electrospinning, which is a well-known fiber production method, is unfortunately hardly applicable for collagen as the current process often leads to irreversible denaturation. Compatible electrospinning processes are being introduced, but other methods are also used and investigated such as fiber extrusion from collagen solutions and isoelectric focusing. [17]

Living tissue substitutes The inherent capacity of cells to synthesize matrix has led to the development of therapies involving scaffold-free tissue engineering. The living substitute is formed by cell-cell and cell-ECM contacts. Figure 1.3 (g) shows a living tissue substitute produced *in vitro* using primary human skin fibroblasts. [3]

1.2 Surgical sealants and hemostatic agents

Hemostasis is a physiological process in the body by which blood loss from a damaged vessel is stopped or limited. It involves vascular, platelet and plasma factors:

- The vascular factors decrease blood loss through local vasoconstriction and the compression of secondary blood vessels.
- The platelet factors: The rupture of a vessel leads to the platelets' adhesion to the injured vessel subendothelial collagen. This leads to the platelets' activation. Platelets provide surface for the assembly and activation of coagulation complexes, and the formation of thrombin. The latter is responsible for the transformation of fibrinogen into fibrin, which is a fibrous protein involved in the coagulation of blood. [1] Fibrin filaments aggregate around the platelets and combined, they form the blood clot.
- Plasma factors interact to form thrombin, which transforms fibrinogen into fibrin.

This series of events, represented in Figure 1.5, help seal back the injured vessel. [18] [19]

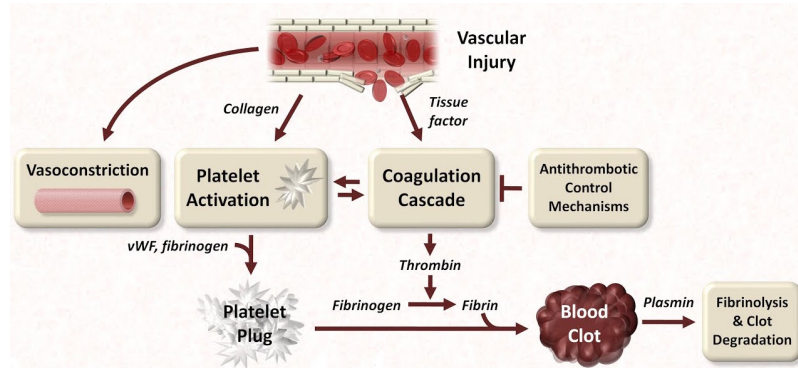


Figure 1.5: Major components and steps of hemostasis [20]

In many internal bleeding injuries, the body's inherent hemostatic mechanisms are insufficient to rapidly stop bleeding, requiring certain materials or technologies to compensate. In contrast with externally accessible injuries, treatment of internal hemorrhages requires the transfusion either of blood or of hemostatic components: platelets, fibrinogen and coagulation factors. This transfusion of hemostatic components accounts for significant challenges in terms of availability, cost or performance variability, which is why research endeavors are focused on developing materials which can mimic and amplify the hemostatic mechanisms. [2]

1.2.1 Types of sealants and hemostatic agents

A wide variety of materials have been developed to help stop bleeding, accelerate the promotion of clotting and prevent leaks. Requirements for such surgical sealants include:

- Strongly bondable to tissue
- Nontoxic agents and degradation products
- As tough and pliable as natural tissues
- Bioabsorbable

Such sealants can be found in many forms, such as pads, powders, foams and glues. The latter are liquids and will cure when applied to form a firm gel. If so, they should rapidly cure under wet physiological conditions, as additional requirement. [21] Depending on the location and type of injury, some agents will be more suitable than others.

Here below are examples of different types of sealants and hemostatic agents, naturally-derived or synthetic:

Glues Fibrin is a critical component for the forming of the hemostatic clot as explained. Therefore, it has become an important material in hemostatic applications. [2] Fibrin glue is based on the natural clotting process as outlined in Figure 1.5. It consists of two components, one containing human fibrinogen, while the other contains thrombin. When applied, the two solutions will mix and thrombin will promote the conversion of fibrinogen into fibrin molecules. These will aggregate into a fibrin clot. The concentration of the fibrinogen determines the strength of the clot, while the amount of thrombin controls the rate of clot formation. These glues have a high degree of tissue adherence due to electrostatic interactions, hydrogen and covalent bonding, and can be applied to heterogeneous injury sites because of their form-filling nature. [2] They have been applied in many surgical fields, cardiovascular surgery being the main one. [21]

A gelatin-based glue, gelatin-resorcinol-formaldehyde (GRF) glue, is used as surgical hemostatic agent as well (GRF glue, Cardial, Technipole, Sainte-Etienne, France). It is composed out of two solutions, a gelatin (G) and resorcinol (R) solution, with a formaldehyde (F) and glutaraldehyde solution. The latter reacts not only with the first solution, but also with the amino groups of tissues, creating bonds between the resulting gel and the tissue. However, its use is controversial due to the possible carcinogenicity and mutagenicity of formaldehyde. [21]

In addition, albumin-based glues also exist. In one of them, BioGlue[®], bovine serum albumin and glutaraldehyde are mixed in a cartridge. It has been used in cardiac, vascular, pulmonary and tissue repairs surgery. Another one, ArterX[®], consists of bovine serum albumin and polyaldehyde. This one is used as sealant around anastomotic sites of synthetic arterial bypass grafts and patches.

Finally, a lysine-derived urethane adhesive, TissuGlu[®], also exists and is applied to the abdominal wall for instance. [21]

PEG-based hydrogels Polyethylene glycol (PEG) monomers can rapidly form hydrogels and crosslink with tissue proteins within seconds, without being exothermic. [22] PEG-based sealants are flexible and firmly adherent, even on wet tissue. One such product, DuraSeal[™], is composed of two separated solutions, PEG-ester and trylysine amine, mixed together *in situ*. They react and form the hydrogel. It is used to reinforce the prevention of cerebrospinal fluid leakage during dural repair procedures. [23] Another product, CoSeal[®], is a synthetic *in situ* polymerizing sealant composed of two PEG derivatives: tetrafunctional glutarylsuccinimidyl ester PEG (NHS-PEG) and four-armed thiol PEG (SH-PEG). They react together to form crosslinked polymers in less than 5 seconds, which then swell approximately 400 % over 24 hours. It is used in vascular reconstructions. [21] [24]

Oxidized-cellulose-based hemostatic agent Cellulose is a homopolysaccharide of glucopyranose, polymerized through β -glucosidic bonds. [2] The use of oxidized cellulose was first reported during World War II and has since then been established as a hemostatic agent. Prior to oxidation, the cellulose can either be regenerated to form organized fibers or remain with unorganized fibers. The fibers are then treated with dinitrogen tetroxide, which oxidizes the hydroxyl groups into carboxylic acid groups, yielding a polyuronic acid. [25] The acidic pH generates platelet activation, initiates the coagulation mechanisms and creates conditions in which most bacteria cannot survive. [2] [25] However, the acidic pH has also been reported to cause an inflammatory response. [26]

Gelatin-thrombin hemostatic matrix Another agent, FloSeal[®], consists of crosslinked gelatin granules and human thrombin. The crosslinking of the gelatin is performed with glutaraldehyde and the thrombin is added to the matrix. The gelatin granules swell to produce a tamponade effect and reduce blood flow, while the high concentration of human thrombin converts fibrinogen into fibrin, accelerating clot formation. [27] Such agent is used in various surgical operations, including neurosurgery. [21]

Collagen hemostatic sheets Several collagen sheets covered with various coatings are used as hemostatic agents. For instance, some of them are coated with human fibrinogen and thrombin. [21] Another sealant, Hemopatch[™], consists of a collagen sheet covered with NHS-PEG, described in more details here below.

HemopatchTM Sealing Hemostat

HemopatchTM is a flexible, resorbable, collagen-based hemostatic and sealing pad (Figure 1.6). Hemostatic means that it is capable of stopping bleeding and inducing coagulation, while sealing refers to its ability to provide a liquid-tight barrier. It is used by surgeons to help stop bleeding during surgery. The collagen pad is covered on one side with a thin layer of NHS-PEG, which favors tissue adhesion.¹ HemopatchTM therefore provides its performance through a dual mechanism. When the pad is applied on a bleeding area, the NHS-PEG coating first enables rapid adherence to the tissue through electrophilic crosslinking with proteins on tissue within seconds, while the collagen is responsible for the formation of a fibrin clot and inducing hemostasis. [1] The collagen matrix also plays a role in absorbing the present fluids. Finally, the word “resorbable” refers to the fact that implanted collagen is degraded through native enzymatic pathways without any toxic response, which is why collagen is often used in the field of wound healing. [28]

The sealing of the bleeding surface by the NHS-PEG coating creates a matrix of platelets aggregation and initiates the release of coagulation factors. Together with plasma factors, these enable fibrin formation. After hemostasis is achieved, the pad can be left in place and is resorbed in 6 to 8 weeks. [29]

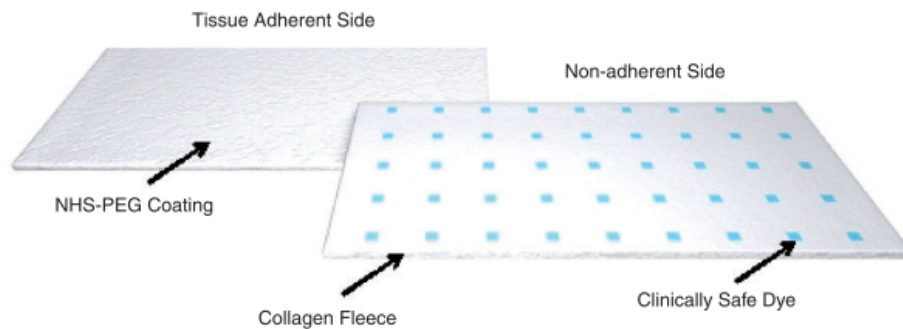


Figure 1.6: HemopatchTM is a polyethylene glycol (PEG)-coated collagen pad designed for hemostasis and sealing. [30]

HemopatchTM's collagen pad - before the NHS-PEG has been applied - is the collagen structure characterized in this thesis. It is derived from bovine epidermis, types I, III and V collagen. [22] It is likely that, as mentioned in literature, the collagen fibrils themselves are a mixture of the different collagen types. They are then called heterotypic fibrils, as opposed to homotypic fibrils which are composed of only one type. [3]

¹NHS-PEG stands for pentaerythritol polyethylene glycol ether tetra-succinimidyl glutarate or N-hydroxysuccinimide (NHS) functionalized polyethylene glycol (PEG).

1.3 Porosity characterization

Highly porous implantable devices are necessary for a wide range of clinical indications, such as hemostasis and wound healing. Pores are an essential consideration in the development of such scaffolds; the scaffold's structure and porosity will have a tremendous influence on its functionality *in vivo*.

In tissue engineering applications, a highly porous, permeable scaffold is required to provide enough space for mass transport, neovascularisation and to act as a template for the new tissue formation. [13] For optimal bioactivity, pores should be large enough to allow cell migration, nutrients diffusion and waste removal. On the other hand, pores should be small enough to promote cell attachment; if pores are too large, the decrease in surface area will limit this adhesion. However, too small pores could potentially restrict cell attachment and their differentiation. [3] [31] This puts into perspective the complexity of

- determining which porosity (e.g. pore size, homogeneity, pore interconnectivity) would be adequate according to the application,
- designing this particular porosity and choosing the correct parameters according to desired porosity.

As a first step, characterization of the porosity of a porous scaffold is key in order to be able to better understand the scaffold's behavior *in vivo* and systematically optimize it.

Porosity definition and quantification

The first question which arises when trying to characterize a porous structure is how to define and quantify porosity. It appears that not one definition can apply, but depending on the characterization method, a different definition is used. This section presents different parameters found in literature and used to characterize scaffolds' porosity, and in particular scaffolds made of collagen or other soft biological materials. Methods used for stiff materials such as metals or ceramics will not be mentioned in this thesis.

Relative density A first idea of the porosity of a collagen scaffold can be obtained through a measurement of its relative density:

$$\frac{\rho^*}{\rho_s} \tag{1.1}$$

where ρ^* is the density of the scaffold and ρ_s is the dry density of solid collagen. This gives the relative occupied volume or occupied volume fraction of the scaffold. To calculate ρ^* , the scaffold's diameter and height can be measured with a laser micrometer, for instance. [13]

Equivalent cell diameter A collagen scaffold, such as the one shown in Figure 1.7, can be considered as a 3D-cellular solid. A cellular solid consists of an interconnected network of solid struts or plates forming edges and faces of cells. [32] In particular, a fourteen-sided polyhedron - a tetrakaidecahedron - has been used many times in literature to model the geometry of collagen scaffolds. Such a solid is graphically represented in Figure 1.8 and is often used to model low-density foams or freeze-dried materials as it packs to fill space, it has been observed experimentally to approximate the structural features of such materials and nearly satisfies the minimum surface energy condition. These materials' pore morphology have shown an average of 14 faces per pore and nearly tetrahedral vertices. [33] [34] [35]

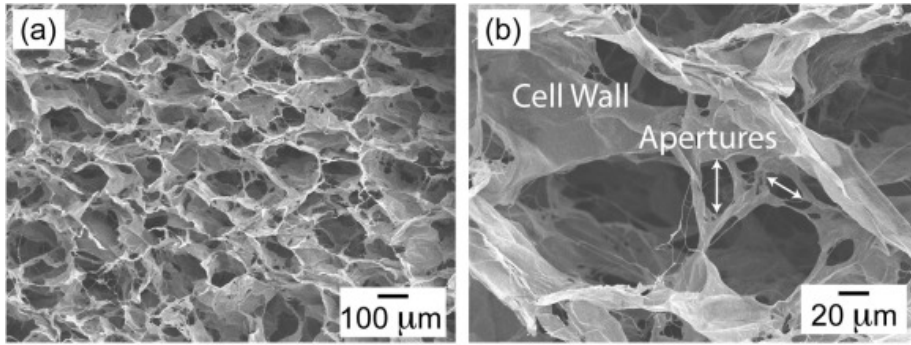


Figure 1.7: Typical SEM image of a collagen scaffold showing its “cellular” structure [13]

By chemically crosslinking scaffolds, slicing them and imaging the gold-sputter-coated slices with SEM, an ellipse-fit method can be used to calculate an equivalent cell diameter. The order of magnitude of the cell wall thickness can also be found - $\sim 1 \mu\text{m}$ was calculated for the scaffold of Figure 1.7 - and this thickness will have an impact on the relative density. [13] Offedu *et al.* (2016) also used SEM images to measure cell wall thickness t_w and strut diameter t_s (Figure 1.8). As shown in Figure 1.8, pore struts are considered the intersection of three distinct pore walls. [34]

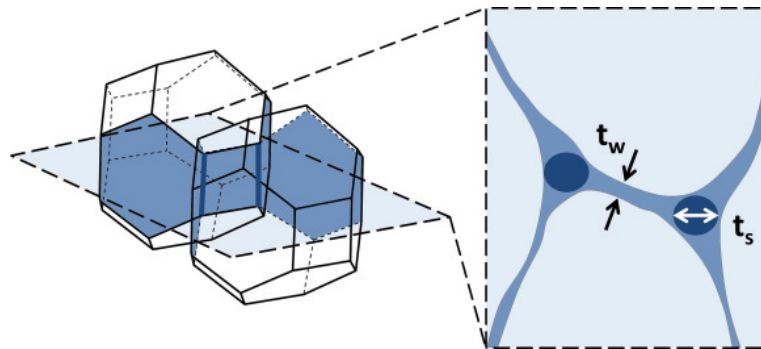


Figure 1.8: Tetrakaidecahedron model used to quantify pore geometry (pore wall thickness t_w and strut diameter t_s) of low-density porous materials [34]

Mean pore diameter Several imaging methods have been used to determine this value:

- Aniline-blue stained samples have been imaged with an optical microscope and then analyzed with a linear intercept program in Scion ImageTM software. It calculated the number of times that a series of parallel lines intercepted a pore wall and used measured distance and angles to construct best-fit ellipses for an average pore cross-section. [33]
- Micro-CT segmented images have also been used to calculate a pore diameter, through ellipse fitting. Pore size can be calculated as the diameter of the circle of equivalent area to each ellipse. The average pore size is then obtained by averaging over all three planes for a given volume. [34] [36]
- The mean pore size has also been measured from SEM images using the MetaVue Image System. [14] [37]
- Finally, multiphoton confocal microscopy, with a 588 nm (blue) laser, has also been used to acquire 3D-microstructural images and obtain the mean pore size. [35]

Specific surface area As mentioned, a scaffold should have a high surface area in order to optimize its bioactivity and particularly cell adhesion. Specific surface area (SSA) is defined as the total surface area of a solid per unit volume (or sometimes per unit mass). [33] Different methods to measure a scaffold's specific surface area have been reported:

- Using a Brunauer-Emmet-Teller (BET) gas adsorption isotherm method with a porosimeter: nitrogen adsorption on the sample is measured as a function of relative pressure. The surface area is determined by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface of the material. [13] [38]
- By CT and 2-photon imaging: the images were thresholded and converted to a 3D surface reconstruction using Avizo Fire software. The total surface area was measured and converted to a SSA. [13]
- Using a cellular solids model, a tetrakaidecahedron can model the geometry of the collagen scaffold, as mentioned before. [33] For a tetrakaidecahedron with edges of circular cross-section, the SSA [μm^{-1}] can be described by the relation:

$$SSA = \frac{3.65}{l} \left(\frac{\rho^*}{\rho_s} \right)^{1/2} \quad (1.2)$$

with l the edge length of the polyhedron in μm . [32] Assuming a constant relative density of 0.5 % and internal volumes similar to spheres of diameter d , the SSA of the scaffold is estimated to be inversely related to the pore diameter d [33]:

$$SSA = \frac{0.718}{d} \quad (1.3)$$

The SSA is thus obtained from the mean pore size, which can be obtained through several microscopy methods as described here above.

- Micro-CT images were also analyzed to calculate the SSA using the Skyscan software CTAn with the 3D analysis function. [34]

The parameters presented here above are of course all related to one another. Potential other parameters exist to characterize and define a scaffold's porosity, but, ultimately, the material's structure and morphology will play a role in what parameters are measurable or not.

1.4 Thermal degradation

Tissues have more and more been heated clinically to treat a multitude of diseases and injuries such as arteriovenous malformations, skin lesions, or to change cornea curvature, among others. Such interventions will have effects on the target tissue but potentially also on the surrounding healthy tissue. This is one of the reasons why thermally-induced changes in collagen have actively been studied, mostly for type I collagen, as it is the most abundant and the one about which most research has been done. In particular, most attention is given to heating which leads to shrinking and denaturation, because many of the therapies treating soft tissues are associated with collagen denaturation. [39] [40]

In the frame of this project, the thermal degradation of the collagen pad has been investigated in view of potentially designing a pad with a heat-controlled pore-size gradient. The side with the native, unmodified collagen would be applied on the wound, showing hemostatic properties as per usual, and the side the furthest away from the wound would be denatured collagen with smaller pores, lower permeability and thus more likely to prevent leaking. Collagen scaffolds with a gradient-pore structure have already been prepared with ice particulates. Using heating to shrink part of the collagen or obtain a pore-size gradient could potentially be an alternative. [14] [37]

1.4.1 Collagen degradation and denaturation

Protein denaturation is the alteration of its biological, physical or chemical properties by some kind of disruption of its structure and caused by an external stress, such as heat, a chemical agent or an enzyme. [41] As the stability of the collagen triple helix greatly depends on hydrogen bonds as explained in Section 1.1.1, collagen will be altered if those hydrogen bonds break, both in terms of structure and thus in terms of function. As these bonds are heat-labile, thermal transformations are one way in which collagen can be denatured, by heat-breaking the H-bonds, leading to a random configuration of the tightly coiled triple helix. This phenomenon is known as *denaturation* and this term will further be used to refer to the collagen's heat-induced transformation on a molecular level. [42]

Reversible or irreversible? It appears that the thermal *degradation* of collagen can be reversible and irreversible. Moderate heating can result in the mere local unfolding of the protein, likely due to the breaking of only a small number of consecutive hydrogen bonds, for instance. Upon the restoration of normal temperatures, the protein regains its native structure. [40] This may be due to the formation of additional H-bonds with the water which was set free during heating and is rebound to the protein when temperature decreases again, leading to a partial renaturation. Finally, it is also suggested that collagen renaturation will be greater if the tissue is maintained under load because increased load tends to organize the structure and thereby promote reformation of some of the triple-helix structures. [43] On the other

hand, severe heating can result in an irreversible transformation of the triple helix into a random coiled structure, via the breaking of long sequences of hydrogen bonds, leading to irreversible degradation. [40]

Speaking strictly of collagen thermal *denaturation* as opposed to degradation, researchers still seem to disagree on whether collagen thermal denaturation is reversible [44] or irreversible [40] [45].

Variability in thermal denaturation Collagen denaturation is highly influenced by the specific composition of the molecule - for instance, by the amino acids present which will allow the formation of more or less hydrogen bonds - but also by post-translational modifications, the molecular organization, the hydration state of the fibers and the environment. [42] In terms of amino acids, the stability of collagen increases as the concentration of hydroxyproline (Hyp) increases in an α -chain. [40]

In addition, crosslinking, whether chemical, physical or biological, is very often introduced exogenously into the molecular structure of collagen scaffolds in order to tune not only mechanical but thermal properties as well. [3] This is the case because the naturally-occurring crosslinking - mediated by the lysyl oxidase - does not occur *in vitro*, which often leads to insufficient strength or collapsing under the pressure of the tissues *in vivo*. [46] The most commonly used chemical crosslinking agents are aldehydes such as glutaraldehyde, isocyanates and carbodiimides. Each crosslinking method leads to a certain degree of structural and mechanical stability, attributed to the different crosslinking mechanisms, concentration and exposure time. [47] This will affect the denaturation temperature. For instance, crosslinking with glutaraldehyde will increase the denaturation temperature from around 65 to 100 °C.

It has also been argued that an increase in denaturation temperature with crosslinking actually occurs because of a reduced water content. In fact, the crosslinks reduce the separation between molecules leading to a loss of water between the molecules. For instance, the denaturation temperature of collagen in bone is very high - around 155 °C - because of the lower water content, compared to other tissues. [48] Thus, denaturation temperature decreases with increasing hydration state, while the denaturation rate increases with increased hydration at a given temperature. [40]

Ultimately, these different parameters affecting collagen denaturation make it quite difficult to compare collagen denaturation between different scaffolds.

1.4.2 Thermal degradation characterization methods

Different parameters have been used to measure the heat-induced modifications of collagen, including shrinking and changes in enthalpy.

Hydrothermal isometric tension (HIT) testing Initially, denaturation was measured by making use of the fact that denaturation causes the tissue to shrink in a narrow temperature range around the denaturation temperature. The procedure involves clamping the sample in the load cell of a tensometer and heating it at a rate between 1 and 10 °C/min. The force necessary to maintain the collagen sample at a fixed dimension is monitored. Denaturation is assumed to occur when there is a sudden increase in the force. This method is limited as it only characterizes thermal degradation via dimensional changes on a macroscopic scale and as the load may impact the contraction of the specimen, yielding an invalid denaturation temperature. [40] [49] Also, it assumes that the molecular *denaturation* is intrinsically linked to the physical shrinking of the collagen.

Differential scanning calorimetry (DSC) This standard bulk thermoanalytical technique has been validated and has become a standard to study collagen denaturation. This phenomenon is accompanied by the absorption of heat, giving rise to an endothermic peak over the denaturation temperature range. Using this technique, it is possible to compare the stability of different collagen samples (as a function of different types of crosslinking for instance [46]), as long as a few considerations are taken into account. Such considerations are included in an article by Loke *et al.* (1995), regarding the validation of the use of DSC to evaluate the denaturation temperature of collagen. [49] It provides an overview of the different parameters which could have an impact on the experimental result and, specifically for collagen or biological samples, in view of creating an experimental technique as common as possible. They discuss instrumental considerations such as calibration, heating rate, the type of pan used and the tightness of the crimp, as well as sample considerations such as wet or blot-dried samples and the impact of crosslinking prior to DSC.

For instance, an important parameter they analyzed is the influence of the heating rate on the denaturation endotherm. They observed that peaks shift to higher temperatures as the heating rate increases from 1 to 10 °C/min, and that the height of the peak and the area under the peak increase as well, as depicted in Figure 1.9. This could be due to “failure to attain thermal equilibrium at faster heating rates”, as collagen is known to denature more slowly than other proteins. This is why thermal studies on collagen should be performed at rates as slow as possible, to reach a denaturation profile which is as close to the equilibrium one as possible. However, the time taken for each analysis as well as reproducibility have to be considered, which is why they suggest using a rate of 5 °C/min as a compromise. [49]

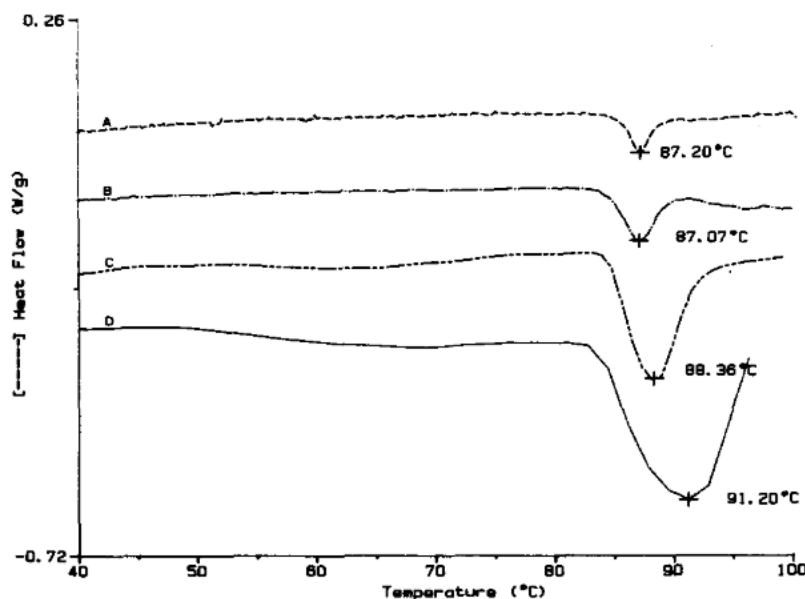


Figure 1.9: Effect of the heating rate on the denaturation endotherm of a glutaraldehyde-fixed porcine pericardium measured with DSC: A: 1 °C/min, B: 2 °C/min, C: 5 °C/min, D: 10 °C/min [49]

Overall, in scientific literature analyzing the denaturation of collagen, the temperature is often raised up to no more than 100 °C at a constant rate of 1 to 10 °C/min, although it is not recommended to go above 5 °C/min as experimented by Loke *et al.* [40] [49]

Microthermal analysis This technique combines atomic force microscopy (AFM) with thermomechanical analysis by replacing the standard AFM probe by a thermally-controlled probe. The thermomechanical response is characterized by recording the probe deflection as a function of the probe temperature. This is known as localized thermomechanical analysis (L-TMA). Before 2011, it was mainly used to analyze polymers and, in 2011, it was considered as a novel approach to use it to understand thermomechanical properties of collagen. The advantages of microthermal analysis over DSC include the ability to perform localized thermal measurements, to obtain spatially-resolved information, such as the imaging of the D-period and its shrinking, inaccessible through bulk techniques such as DSC, and therefore to avoid destroying the sample completely. However, one limitation of this approach is the need to use high heating rates of around 20 °C/min. Reducing the heating rate would lead to a more equilibrated thermal state, but it would increase the measurement time to a few hours, leading to a decrease of the stability of the measurement due to the displacement of the probe from its original point of contact with the sample. [50]

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

2.1.1 Collagen-based porous material

Collagen sheets measuring 210 x 297 x 2 mm were used. The collagen is derived from bovine epidermis from Argentina, composed of collagen types I, III and V. They are produced by a subcontractor and are unsterile. The same batch was used for all experiences (lot number 1401779). These collagen sheets are the collagen material used in HemopatchTM as explained in Section 1.2.1. The pads are produced through freeze-drying and are not chemically crosslinked. No more information is available on the manufacturing process of those sheets.

For some thermal degradation experiments, HemopatchTM's collagen was compared with another collagen pad also used as hemostatic agent, Duraform[®] Dural Graft Implant. The collagen is also bovine-derived but no information is provided about its geographical origin.

2.1.2 Chemicals

Glutaraldehyde ($\text{OHC}(\text{CH}_2)_3\text{CHO}$) in solution (25% in H_2O) from Sigma-Aldrich was used as fixative. Phosphate-buffered saline (PBS) tablets were also provided by Sigma-Aldrich. Poly(allylamine hydrochloride) (PAH)-rhodamine was used as chemical dye (MW 150 KD, 10.47 g/L, excitation wavelength = 551 nm, emission wavelength = 573 nm). It was purchased from Surflay Nanotec GmbH. Pelleted paraffin wax with a congealing point of 56 °C from Laborimpex was used to rigidify the collagen sample.

Suspensions containing standard size polystyrene (PS) particles from Thermofisher (Duke StandardsTM 2000 Series Uniform Polymer Particle and 3K/4K Series Particle

Counter Standards) were used. The suspensions contained particles with a diameter of 2 μm (Catalog number 4K-02), 5 μm (Catalog number 4205 A), 10 μm (Catalog numbers 4K-10 and 4210A), 20 μm (Catalog number 4220A) and 30 μm (Catalog number 4230A).

2.2 Visualization techniques

2.2.1 Optical microscopy

As first visualization method, optical microscopy was used to investigate the collagen pad structure. Two different optical microscopes were employed: a Leica M80 stereomicroscope and a Leica DM 2500 P polarized light microscope. To enhance the contrast of the sample, the collagen was gold-coated with a 30 second cycle at 28 mA using an Agar Auto Sputter Coater.

2.2.2 Scanning electron microscopy

To investigate the surface morphology of the collagen pad, scanning electron microscopy (SEM) images were taken using two scanning electron microscope models: a JEOL 7600F (lanthanum hexaboride or LaB_6 filament) and a FEI Quanta 250 (tungsten filament). The samples were sputter coated with a 15-nm-gold layer (80 mA) using a Cressington 208HR High Resolution Sputter Coater or with a thin layer of gold with a 30 second cycle at 28 mA using an Agar Auto Sputter Coater to reduce charging, produce conductive surfaces and thus enhance the contrast of the sample being imaged. For the imaging, the acceleration voltage was held between 1 and 5 kV with both SEM equipments.

In order to rigidify the sample to be able to cut cross-sections and visualize the center of the pad, three different fixation methods were used on the collagen pad:

1. Water and cryogenization with liquid N_2 The sample was placed in the cryogenization chamber with liquid N_2 of a microtome (Leica EM FC6). The sample was briefly immersed in water, then cryogenized in the microtome chamber brought to -50°C . Cross-sections of 10 or 15 μm were cut and were laid flat on a surface. Afterwards, these cuts were heated at 50°C to rapidly evaporate the water contained in the cross-section. The imaging was done at least 24 hours afterwards, to ensure that all remaining water could evaporate.

2. Paraffin wax Paraffin wax was heated at 70°C to completely melt it, cooled down to 50°C and then embedded in the collagen pad, just by immersing the pad in the paraffin. The pad was taken out of the paraffin, cooled down at ambient conditions to solidify. Cross-sections of the pad of 10 μm were cut using the microtome; thicker

ones could not be made because the cut would simply roll onto itself. Afterwards, the paraffin was removed by applying a drop of toluene, which dissolves the paraffin.

3. Glutaraldehyde A glutaraldehyde solution was used to chemically crosslink the sample by creating bonds between amine functions. First, a PBS solution was prepared by dissolving a PBS tablet in 500 mL of demineralized water, to obtain a pH of 7.4 and a concentration of 0.01 M. Then, 1 mL of a 25-%-concentrated glutaraldehyde solution was added to 9 mL of the PBS solution. This formed a 2.5-%-concentrated glutaraldehyde solution.

The sample was immersed for 24 hours in the solution in the fridge. Afterwards, the sample was dehydrated in ethanol-water baths of increasing ethanol concentration: 10, 30, 50, 70, 80, 90, 100 %, leaving the pad for 1 minute in each bath.

2.2.3 Confocal microscopy

Four different samples were characterized through confocal laser scanning microscopy, using a Zeiss LSM 710/axio Observer Z1/Airyscan microscope, equipped with a Plan-Apochromat 63x/1.4 oil DIC objective. A laser line with a wavelength of 561 nm (yellow-green) was used to illuminate the sample. The emission was taken between 566 and 685 nm in confocal mode. The reading was obtained with a GaAsP photomultiplier tubes detector. Image stacks were acquired and 3D-reconstructed using ImageJ.

Sample preparation

Four collagen samples were prepared, as displayed in Table 2.1. The samples were cut from a collagen sheet with razor blades and were all around 1 mm thick. Three out of the four samples were prepared the day before visualization. Rhodamine PAH was used as chemical dye. Three different solutions of 1 mL were prepared, each with a different solvent: acetone, ethanol or distilled water. Diluting the dye 100 times, each solution had a final rhodamine concentration of 0.1 mg/mL.

Table 2.1: Samples prepared for confocal microscopy

Sample number	Solvent	Evaporation
1	Ethanol	Yes
2	Acetone	Yes
3	Water	Yes
4	Water	No

The three samples were placed in the three respective solutions for 20 minutes. Afterwards, each sample was placed in two consecutive baths of its solvent (pure) for 1 minute each in order to remove the excess dye. Finally, the samples were placed on individual glass slides and dried at ambient air, until all liquid had evaporated.

The samples were then placed on new individual glass slides, covered with a glass cover slip and held together with nail polish. They were kept overnight at ambient temperature in aluminum to avoid the degradation of the fluorescence.

The fourth sample was prepared right before the confocal microscopy was performed in order to image the sample *in situ*. Again, a 1 mL solution was prepared, with a 0.1 mg/mL rhodamine final concentration, using distilled water as solvent. The sample was placed in the solution for 20 minutes in the dark then rinsed in two baths of distilled water for 1 minute each. Finally, it was placed in between a glass slide and a cover slip and maintained with nail polish, just like the other three. This sample was imaged right away, allowing as little evaporation of the water as possible to occur.

2.2.4 X-ray micro-computed tomography (Micro-CT)

A dry collagen sample was imaged using a Phoenix NanoTom S (GE Measurement and Control Solutions, Germany). A molybdenum target was applied. The system was operated at a voltage of 70 kV and a current of 60 μ A. The exposure time was 500 ms and 2400 images were acquired over 360 °, with a frame averaging of 3 and image skip of 1. The collagen pad was examined at a 1 μ m³ isotropic voxel size. 3D-analysis was done with 3D.Suite CTAn software and 3D-reconstruction with 3D.Suite CTVox software. A 3D-Gaussian filter with radius 1 was applied (round kernel) and a Multilevel (Otsu method) thresholding was done with 3 threshold levels. Afterwards, a closing operation in 3D-space was applied (radius 1). Black and white 3D-speckles with a volume below 30 voxels were removed.

2.3 Relative density measurement

The relative density of the collagen scaffold was calculated according to Equation 1.1:

$$\frac{\rho^*}{\rho_s}$$

where ρ^* is the density of the scaffold and ρ_s is the dry density of solid collagen, equal to 1.3 g/cm³. [34]

The density was measured on ten collagen samples, by measuring their mass, surface area and thickness. Their thickness was measured using a motorized low force high precision gauge (Mitutoyo Litematic VL-50S).

The relative density was also calculated after heating the same ten collagen pads in an oven (WTB Binder) at 225 °C for 4 minutes. The pads were placed on aluminum crucibles which were already hot beforehand. Again, the ten samples' mass, surface area and thickness were measured. Both the surface area and the thickness were measured using an optical stereomicroscope (Leica M80).

2.4 Particle size analysis

Particle size analysis is an analytical technique used to characterize the size of particles in a liquid. It provides information about the particles in the suspension or emulsion such as their number, size, shape and size distribution.

This analytical method was used to characterize the collagen pas as a humid membrane and provide a general overview of the pores' distribution, by analyzing the size of the particles which would be retained by the pad. Two different particle-size-analysis instruments were used.

2.4.1 Occhio

Occhio Ipac 2 is a particle size counter based on dynamic image analysis (Figure 2.1). Pictures of the tested liquid are taken over time and analyzed in real time by a software. A 10 Megapixel camera is set on the opposite side of the blue light source (monochromatic LED with a 440 nm wavelength) with the observation cell in between. A syringe pumps the liquid, which is forced to flow in between the two glass plates of the microflow cell at a controlled flow rate. The cell has a defined thickness and can be adjusted to accommodate to the particle size range. This analytical method is based on 2D-image analysis. Therefore, an accurate control of the cell thickness is required to calculate the volume analyzed on each picture, as the volume analyzed per frame is equal to the area of the picture multiplied by the cell thickness. Finally, the total analyzed volume is computed by software as the sum of the volumes of each recorded image.

The flow rate has to be sufficient to prevent the overlap of two images, in order to capture the real number of particles and not count a same particle twice for instance. However, it has to be below a critical value to avoid blurry particles when the picture is taken, which would provide wrong information about the number of particles or their size. To ensure a proper flow rate, it has to be determined in accordance with the number of frames (images) taken per second (FPS). [51]

The Callisto 3D software analyzes the recorded pictures in real time, and provides an analysis of the size of the particles present in the liquid, as well as the size distribution, using the principle of obscuration. A particle will be detected if the difference between its gray scale and the background is superior to the chosen threshold value.

Three suspensions containing PS standard size particles were diluted in Milli-Q water. Each suspension contained particles with one diameter: 2, 5 or 10 μm . Each suspension had a respective concentration as indicated in Table 2.2. The microflow cell used had a thickness of 50 μm , which allows to detect particles with a diameter between 0.344 and 50 μm . Each of these three solutions were analyzed with Occhio, by analyzing three individual replicates of 0.3 mL for each solution.

Three times 0.3 mL Milli-Q water were also analyzed with Occhio to make sure no particles were originally detected in the Milli-Q water used to dilute the suspension.

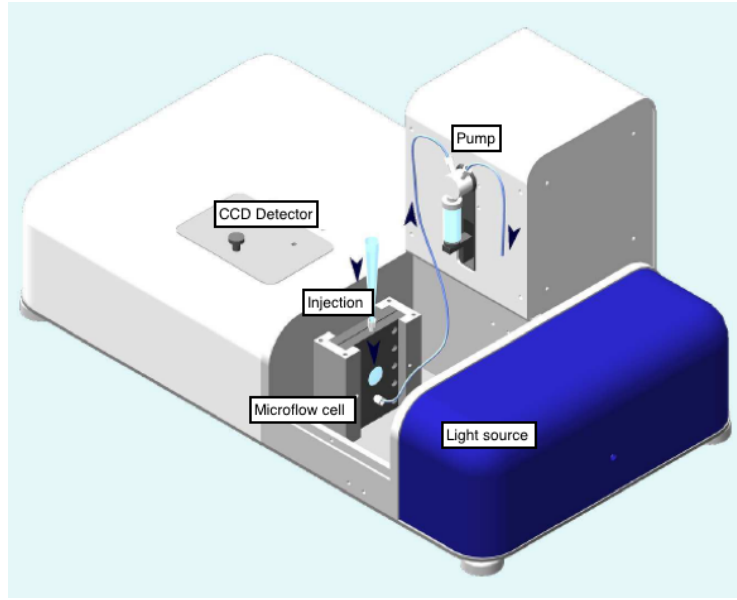


Figure 2.1: Occhio Flow Cell Device [52]

Table 2.2: Concentration of the suspensions containing standard size particles for analysis with Occhio

Diameter of the particles in the suspension [μm]	Concentration [particles/mL]
2	$2.5 \cdot 10^5$
5	$2.5 \cdot 10^5$
10	$1 \cdot 10^5$

20 mL of the suspension containing 2- μm -diameter particles were passed through a 5 x 5 cm² collagen sample using a filtration set-up as shown in Figure 2.2. 0.3 mL of the obtained filtrate (16 mL) was again analyzed with Occhio. This was done three times, by filtrating three times 20 mL through three different collagen samples. All three filtrates were analyzed with Occhio.

2.4.2 Parenteral Particle Counter

The second particle size analyzer used is the APSS-2000 Parenteral Particle Counter (PPC), provided by Particle Measuring Systems[®] or PMS. This analyzer also counts particles based on the principle of obscuration, as explained in Section 2.4.1. A syringe pumps 5 mL of liquid with a flow rate of 10 mL/min, and through obscuration, PPC provides the detected number of particles per mL with a diameter above 2, 5, 10, 20 and 25 μm . The system pumps 5 mL 4 times (20 mL total), discards the first one (5 mL) and averages the three others. It allows to detect particles with a diameter between 1.5 and 125 μm .

A single suspension was prepared, containing standard size particles with a diameter of 2, 5, 10 and 20 μm . The concentration of the suspension is shown in Table 2.3.

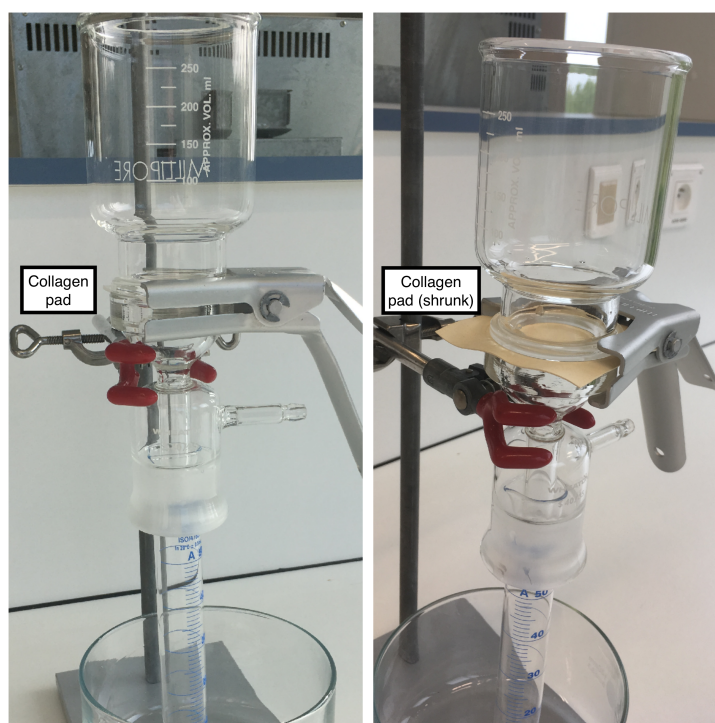


Figure 2.2: Set-up for filtration of suspension through collagen pad

Three samples of 20 mL were analyzed with PPC.

Table 2.3: Concentration of the suspension containing standard size particles for analysis with Parenteral Particle Counter (PPC)

Diameter of the particles in the suspension [μm]	Concentration [particles/mL]
2	$5 \cdot 10^4$
5	$5 \cdot 10^3$
10	200
20	40

Afterwards, three times 35 mL of the suspension were passed through three different $5 \times 5 \text{ cm}^2$ collagen samples using a filtration set-up as shown in Figure 2.2. 31 mL of filtrate was obtained for each sample. 20 mL per obtained filtrate were again analyzed with PPC.

Finally, three $21 \times 21 \text{ cm}^2$ collagen samples were heated at 225°C for 4 minutes in an oven. After shrinking, 35 mL of the suspension was filtered through each shrunk pad. The three filtrates (31 mL) were analyzed with PPC.

2.5 Thermal analysis techniques

2.5.1 *In situ* heating

In order to visually characterize the thermal degradation of the collagen pad, a heating stage was installed, allowing to heat the sample while visualizing it either with the optical microscope (transmission mode) or the SEM. On the SEM (FEI Quanta 250), a FEI WetSTEM heating stage was installed as shown in Figure 2.3. The sample used was a square of $\sim 4 \times 4 \text{ mm}^2$ as to fit exactly in the circular sample holder as indicated in Figure 2.3, and be maintained as immobile as possible during the heating. A heating rate of $5 \text{ }^\circ\text{C}/\text{min}$ was applied. A Linkam THMS600 heating stage was used for heating a sample on the optical microscope. A heating rate of $10 \text{ }^\circ\text{C}/\text{min}$ was applied. For both microscopy techniques, the sample was gold-coated with a 30 second cycle at 28 mA and heated from $25 \text{ }^\circ\text{C}$ up to when the site being imaged was not recognizable anymore or the sample had moved too much due to the extent of the shrinking.

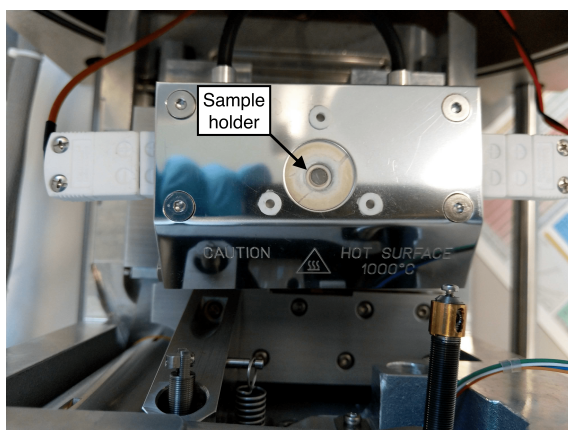
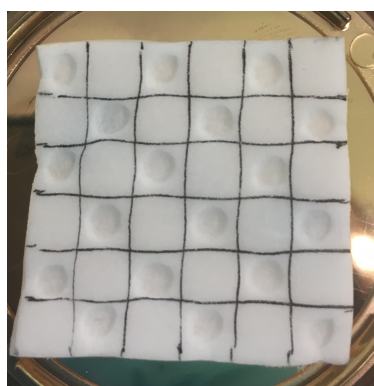


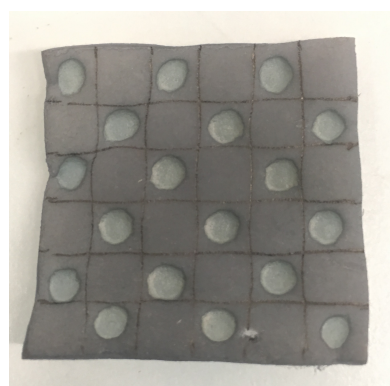
Figure 2.3: FEI WetSTEM heating stage connected to SEM used for *in situ* heating of collagen pad

As an alternative to *in situ* heating, a $3 \times 3 \text{ cm}^2$ collagen sample was cut and a grid of 0.5 cm by 0.5 cm was drawn on it, as shown in Figure 2.4a. In every other square of the grid, $10 \text{ }\mu\text{L}$ of a suspension with standard size particles with a diameter of $30 \text{ }\mu\text{m}$ were dropped (Figure 2.4a). The pad was gold-coated with a 30 second cycle at 28 mA (Figure 2.4b) and one specific square of the grid was then imaged with SEM, particularly focusing on two recognizable spots thanks to the standard size particles.

The sample was heated in an oven at $100 \text{ }^\circ\text{C}$ for 4 minutes, at $150 \text{ }^\circ\text{C}$ for 4 minutes, at $200 \text{ }^\circ\text{C}$ for 4 minutes, at $210 \text{ }^\circ\text{C}$ for 1 minute, at $210 \text{ }^\circ\text{C}$ for 2 additional minutes and at $225 \text{ }^\circ\text{C}$ for 1 minute. In between each heating step, the same square of the sample was imaged with SEM, focusing on the same two recognizable spots each time. The distance between the particles in each of the two spots was measured each time, to follow the evolution of the shrinking.



(a) Uncoated



(b) Gold-coated

Figure 2.4: Collagen sample with 30- μm -diameter particles in every other square of the grid

2.5.2 Visualization of the change in porosity due to heating

In order to visualize the effect of heating and thermal degradation on the porosity of the collagen pad, an aluminum grid was heated in an oven at 300 °C for 10 minutes to ensure the grid reached 300 °C. Afterwards, the hot grid was taken out of the oven and placed on top of a collagen sample for 5 minutes, with a 1-kg-mass on top, to ensure a good contact between the sample and the grid. The sample was gold-coated with a 30 second cycle at 28 mA and then imaged with SEM.

2.5.3 Differential scanning calorimetry (DSC)

The thermal transitions of the collagen pad were characterized by differential scanning calorimetry (DSC) analysis under nitrogen flow of 30 mL/min. A Mettler Toledo DSC1 was used. Two different heating cycles were used, each on two different types of samples: an unmodified sample and a sample which had already been heated in an oven at 225 °C for 4 minutes beforehand. The mass of all samples ranged between 2.0 and 2.5 mg.

Heating cycle 1 Firstly, the sample was held at 5 °C for 5 minutes, then heated at a rate of 5 °C/min up to 300 °C. Three replicates were done.

Heating cycle 2 This was also applied to three replicates. Each sample was first held at 5 °C for 5 minutes, then the following heating segments were applied, all with a rate of 5 °C/min:

- 5 to 110 °C,
- 110 to 5 °C,
- 5 to 230 °C,

- 230 to 190 °C,
- 190 to 240 °C.

2.5.4 Dynamic mechanical analysis (DMA)

Two 5-mm-diameter discs of collagen were used for this experiment. The storage and loss moduli of the collagen pad were measured as a function of temperature in shear mode, using a Mettler Toledo DMA/SDTA861. A schematic representation of this DMA-mode is represented in Figure 2.5. The two samples were placed in between a fixed and a mobile mounting. The mobile mounting oscillates at a certain frequency and with well-defined force and displacement amplitudes.

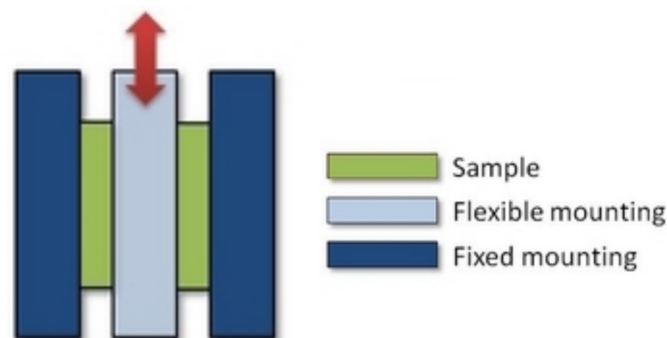


Figure 2.5: Schematic representation of DMA shearing deformation mode [53]

In order to determine the elastic domain of the sample, in which the moduli can be measured, a force-displacement test was performed beforehand. This pre-test revealed that the linear stress-strain behavior of the collagen pad was preserved at least up to 0.1 N and 5 μm . Therefore, the shearing of the sample was done with an amplitude of 5 μm and an upper-force limit of 0.1 N. The shearing frequency was 1 Hz and the heating rate 3 °C/min.

The moduli were measured between 30 and 230 °C.

2.5.5 Thermomechanical analysis (TMA)

This technique allows the measurement of a sample's shrinkage or expansion as a function of temperature, time and applied force. The variation in thickness of a 5-mm-diameter collagen disc was monitored as a function of temperature. A Mettler Toledo TMA/SDTA841 was used in contact mode which means that a force of 0.01 N was applied on the sample during the experiment. The sample was heated from 30 up to 230 °C with a heating rate of 5 °C/min.

2.5.6 Thermogravimetric analysis (TGA)

The evolution of a collagen sample's mass was measured as a function of temperature using thermogravimetric analysis (Mettler Toledo TGA/SDTA851). A 5-mm-diameter collagen disc was used with an initial mass of 1.25 mg. The heating of the sample was done under nitrogen atmosphere (50 mL/min) with a heating rate of 10 °C/min from 30 to 400 °C.

2.5.7 Chemical degradation with temperature

As it is known - and has been observed in this thesis - collagen degrades with rising temperature. However, the following question arises: is this degradation purely physical - due to a decrease in the number of physical cross-links for instance - or does the chemical structure of the material actually change? If the latter is the case, one can also wonder at which level of the structural hierarchy of the collagen the chemical changes occur.

Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (also known as infrared absorption spectrophotometry) is used in this thesis as a first window into the chemical degradation of the collagen pad. IR spectroscopy enables the detection of functional groups present in a sample, by stimulating molecular vibrations. Chemical bonds absorb radiation of the same frequency as their natural frequency. Bond vibrations have a natural frequency in the range of $1.20 \cdot 10^{13}$ to $1.20 \cdot 10^{14}$ Hz, which is in the infrared region of the electromagnetic spectrum. This is why IR radiation is used to pass through the sample. As a specific frequency is absorbed by the sample, this results in less radiation being transmitted, which is what the detector measures. [54] The term "Fourier-transform" refers to the fact that a Fourier-transform algorithm is required to convert the raw data into a spectrum. [55]

In order to analyze the chemical degradation of the collagen pad with increasing temperature, the spectrometer was coupled with a heating stage (Linkam THMS600), and the spectra were recorded every 25 °C, from 25 up to 225 °C. This was used to see whether one or several peaks associated to functional groups shift or even disappear completely as temperature increases. A very thin sample was cut and placed in the closed cell of the heating plate. The thickness of the sample cannot be measured accurately but it can be estimated to 50 μm , as thicker leads to a signal saturation.

Enough time (around 5 minutes) was left in between each temperature interval in order to allow for the sample to be heated homogeneously. A resolution of 2 cm^{-1} was used.

To generate one spectrum, 100 scans were performed by the spectrometer at one temperature. Then, the spectrum was formed by doing the Fourier-transform on all 100 scans. Having a high enough number of scans allows to increase the signal-to-noise ratio (SNR), which improves the spectrum's baseline. The SNR increases as a function of $\sqrt{N}$, where N is the number of scans, thus by increasing the number of scans over a certain measuring time. As $N = 100$, the SNR was increased by a factor of 10. [56]

This chapter presents the results obtained regarding the porosity characterization of the collagen pad, as well as its thermal degradation. The first part presents different visualizations of the collagen pad, which allow to provide a first idea of the pad's structure. The second part involves the pad's porosity quantification. The third and final part addresses the thermal transitions and degradation of the pad.

3.1 Scaffold visualization

The first part of this work aimed at visually analyzing the collagen pad structure of HemopatchTM Sealing Hemostat and gaining a first insight into its morphology, in view of quantifying its porosity accordingly afterwards.

3.1.1 Surface

The morphology of both surfaces of the pad was analyzed by scanning electron microscopy (SEM). As shown in Figure 3.1, the scaffold is constituted of a seemingly high degree of porosity, some thick fibers with a diameter $\varnothing$ of at least 10 μm , and other fibers with a diameter of 1 μm or less. Furthermore, imaging the pad with SEM also showed that no difference is visible between one surface of the pad and the other on the microscopic scale. Therefore, no differentiation was made between both surfaces when imaging the pad. Figure 3.1 also demonstrates the difficulty to define the concept of pore within this 3D-scaffold.

Imaging the pad's surface with SEM at a higher magnitude also allows to visualize the D-period of the collagen fibrils, as displayed in Figure 3.2. The D-period is a pattern created by the tropocollagen molecules which pack together at about one quarter of their length, around every 67 nm. [4] This is the scale of the banding pattern observed, as indicated in the right image of Figure 3.2.

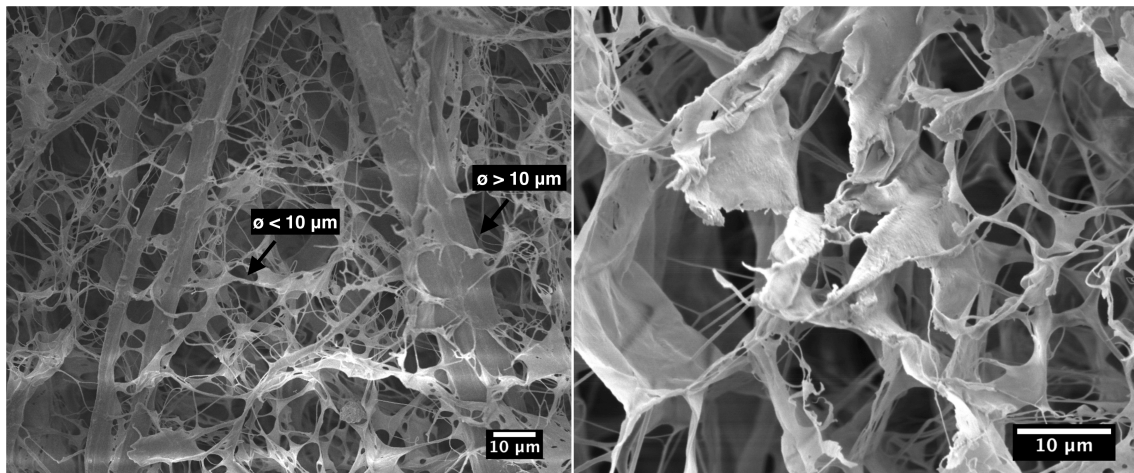


Figure 3.1: Gold-coated collagen scaffold imaged with SEM, showing the morphology of the pad's surface and the different scales of the fibers. The left image shows fibers with a diameter $\varnothing$ larger than $10\ \mu\text{m}$, and others with a diameter much smaller than $10\ \mu\text{m}$. The right image displays the porosity at a little higher magnification.

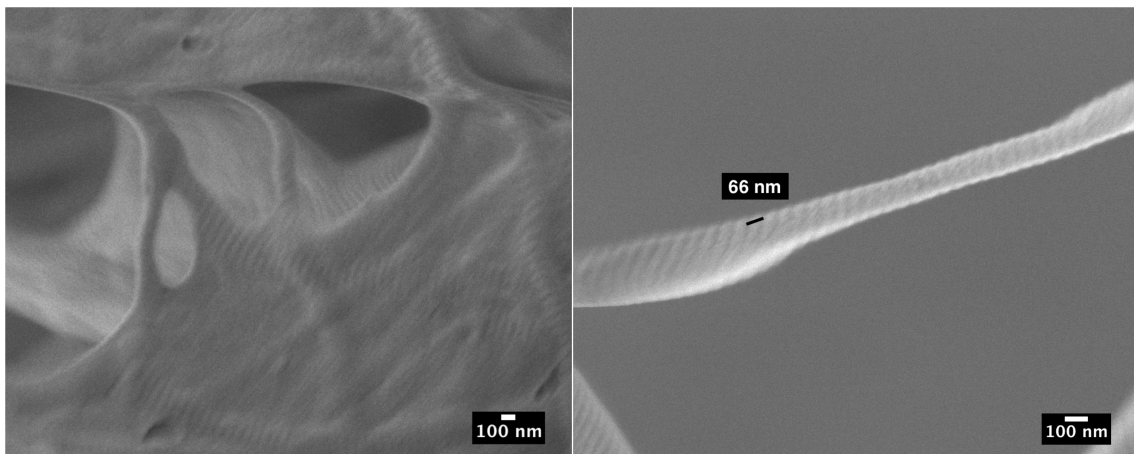


Figure 3.2: Gold-coated collagen scaffold imaged with SEM showing collagen D-period. On the left, small fibers with D-period visible as well as wider collagen surfaces are shown. On the right, the focus is made on one fiber, clearly showing the D-period.

3.1.2 Cross-sections

Scanning only the surfaces does not ensure that the bulk or internal part of the scaffold in between the two surfaces has the same morphology. In order to identify whether the internal part of the pad is different, several attempts were made at cross-sectioning the pad in order to image a cross-section with SEM.

Cutting cross-sections of the bare collagen pad with the microtome proved to be very challenging, due to the high porosity, and flexible nature of the pad, which make it very fragile. Therefore, three attempts at rigidifying the sample were made: cryogenizing the sample under liquid N_2 , embedding the sample in paraffin wax

or chemically crosslinking it with glutaraldehyde. However, neither of these three methods was successful at leaving the sample unmodified when the cross-section was made, and they dramatically altered its morphology as can be seen in Figure 3.3 in comparison with Figure 3.1.

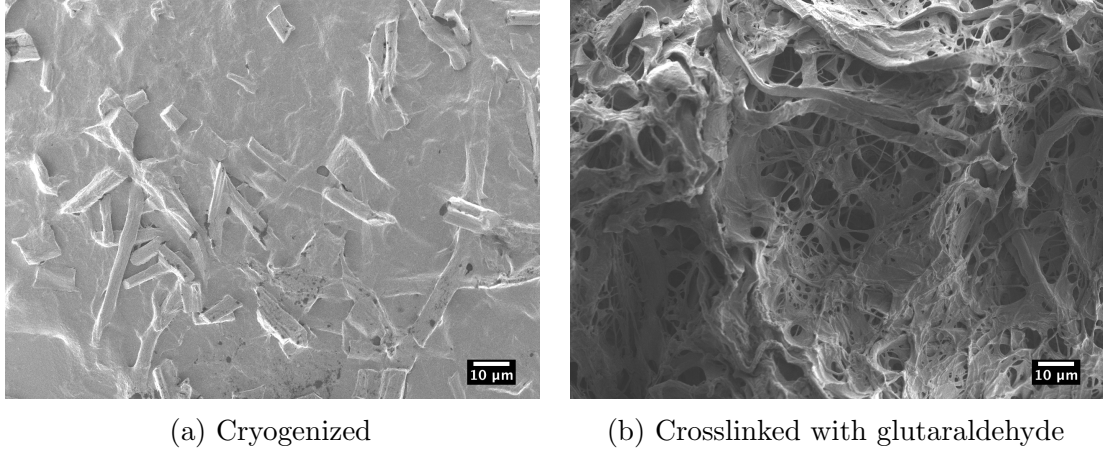


Figure 3.3: Gold-coated collagen scaffold cross-sections imaged with SEM, showing the damage caused to the pad’s structure by the cryogenization (a) and the glutaraldehyde crosslinking (b)

In the case of the cryogenized sample and the paraffin-embedded sample, it was impossible to obtain a flat cross-section as it would either roll onto itself or form chips. Furthermore, the cryogenization seems to generate ice-induced damage in the soft collagen scaffold (Figure 3.3a). For the glutaraldehyde-crosslinked sample, immersing it in a solution and leaving it to dry at ambient conditions inevitably degraded the sample, as seen in Figure 3.3b, even more because the ethanol baths contributed to the sample dehydration, causing it to collapse onto itself. Macroscopically, the sample shrunk in all three directions and was more rigid. As explained in Section 1.4.1, glutaraldehyde is quite often used to crosslink collagen scaffolds, but, unlike here, the crosslinking occurs as part of the initial scaffold fabrication process, not after it has completely dried. Furthermore, the drying occurs under vacuum and therefore faster, avoiding the collapse of the sample.

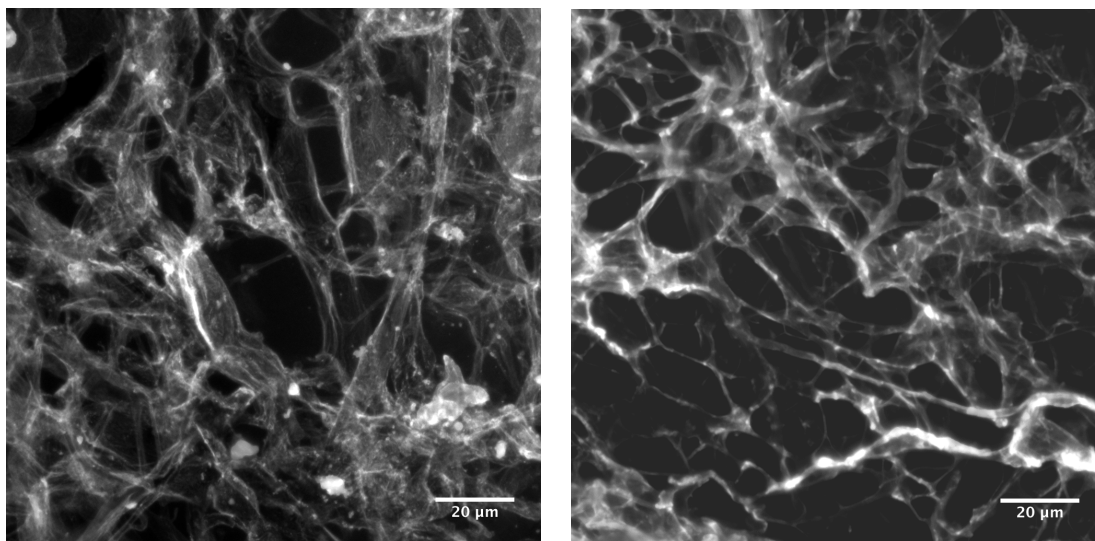
In the end, characterizing the pad with SEM offers the advantage of very clearly showing the scaffold morphology, the range of the pore size as well as the thickness of the fibers, thick and thin. However, it is limited by its capacity to produce 2D-images and only of the surface of the sample since no method was found to properly cut it and characterize its internal composition without altering the scaffold in some way.

3.1.3 3D-visualization

As cutting cross-sections of the pad did not work, 3D-imaging techniques were employed to scan the pad and visualize its inner structure.

Confocal microscopy Stacks of images produced with confocal microscopy provide a 3D-view of the scaffold, as shown in Figure 3.4. The sample in Figure 3.4a was prepared with water as solvent and imaged *in situ* while the sample in Figure 3.4b was prepared with acetone as solvent and dried at ambient temperature before imaging. Both were dyed with PAH-rhodamine but a black-and-white rendering enables to better visualize the contrasts.

The two other samples prepared with ethanol and water as solvents, both dried at ambient air before imaging, could not be imaged, due to the drying stage. In fact, no vacuum drying was available which would have allowed the samples to dry more rapidly. The ambient-air drying probably occurred too slowly, which caused the sample to collapse and the fragile scaffold to break. It is also possible that the drying only partially occurred, leaving the sample humid overnight and completely altering its structure. Vacuum-drying might therefore help to preserve the scaffold's initial structure. This was not an issue for the sample prepared with acetone as it evaporates faster and it therefore did not alter the sample as much.



(a) Water as solvent and imaged *in situ*

(b) Acetone as solvent and dried before imaging

Figure 3.4: Collagen scaffold dyed with PAH-rhodamine and imaged with confocal microscopy

The depth of imaging, and therefore the height of the stack, was influenced by the density of the scaffold; scans were gathered in the stack until nothing was visible anymore and the signal lost resolution. Figure 3.4a shows the scaffold with a depth

of $19.17\ \mu\text{m}$ while Figure 3.4b displays a depth of $28.00\ \mu\text{m}$. Further than that, no visualization of the scaffold was possible anymore.

These two images enable to identify some of the thicker and thinner fibers of the scaffold. However, these stacks show that confocal microscopy does not allow to visualize the clear, sharp scaffold details visible on SEM images such as in Figure 3.1. Due to the diffuse nature of the images, it is difficult to measure pore sizes on them. This was experienced by Tomlins *et al.* (2004) as well. [57] Furthermore, a 3D-rendering is possible but with a depth no higher than $28\ \mu\text{m}$, which does not allow to see the pores very far into the sample, meaning that even the 3D-view is limited with confocal microscopy.

Micro-CT Scanning the collagen pad with micro-CT also provides a 3D-view of its structure, as shown in Figure 3.5. It is important to remember that the collagen sample was examined at a $1\ \mu\text{m}^3$ isotropic voxel size, which means that the fibers with a diameter below $1\ \mu\text{m}$ are not detected and therefore not shown in the visualization. This is why the scaffold architecture seems quite different compared to the SEM images such as in Figure 3.1.

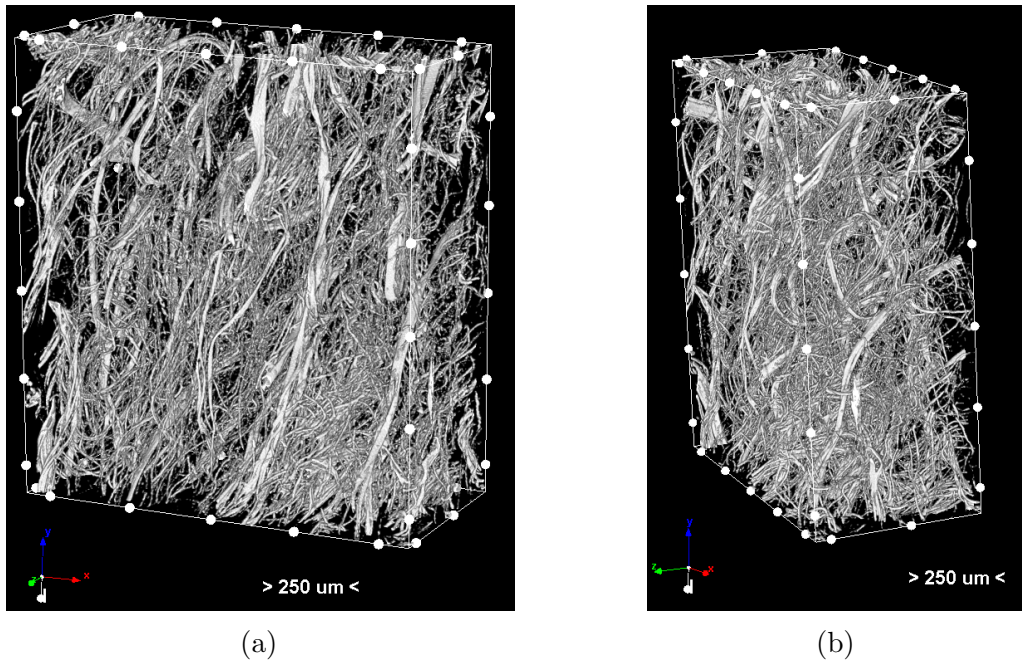


Figure 3.5: 3D-view of the collagen pad obtained through reconstruction of micro-CT scans, showing only the fibers with a diameter above $1\ \mu\text{m}$

The imaged collagen sample had a surface area of $2651\ \text{by}\ 2709\ \mu\text{m}^2$ and a thickness of $2179\ \mu\text{m}$, which is the thickness the collagen sheets have in general ($\sim 2\ \text{mm}$). These dimensions are determined by the 3D-reconstruction software CTVox. Figure 3.5 shows only a part of the 3D-reconstruction, which allows a better visualization of the fibers.

In comparison with confocal microscopy which only shows the scaffold $\sim 20 \mu\text{m}$ into the sample, micro-CT allows a complete 3D-visualization, scanning completely across the sample. On the other hand, it does not show fibers with a diameter below $1 \mu\text{m}$ and therefore provides a less representative visualization of the morphology of the pad. Yet, it allows to retrieve some parameters to quantify the porosity of the sample, as will be discussed in the following sections.

Table 3.1 presents an overview of the three visualization techniques used to image the pad, summarizing their respective advantages and drawbacks with regards to the collagen pad visualization.

Table 3.1: Synoptic table of visualization methods

Method	Advantages	Drawbacks
SEM (Figures 3.1 and 3.2)	High resolution Very well-resolved small fibers D-period visualization	2D-representation Examination of surface pores and fibers only
Confocal microscopy (Figure 3.4)	3D-visualization	Diffuse scaffold Limited depth for imaging Small fibers detectable but not well resolved
Micro-CT (Figure 3.5)	3D-visualization Imaging across the entire sample thickness	$1 \mu\text{m}$ resolution No detection of small fibers

3.2 Porosity quantification

After visualizing the collagen scaffold through different techniques, the next step focuses on characterizing and quantifying the scaffold porosity. This step is key in order to better make sense of the scaffold's behavior *in vivo* and be able to systematically improve it. As discussed in Section 1.3, various parameters can be used to describe a material's porosity: pore size, pore size distribution, and relative density are a few of them. This section presents the ways in which the scaffold's porosity was successfully characterized and through which parameters, but also seeks to explain why some methods failed, which also provides an insight into the collagen pad's architecture.

3.2.1 Relative density

The measurement of $\frac{\rho^*}{\rho_s}$ (Equation 1.1) provides a first idea of the scaffold's porosity, as it gives an indication of the relative occupied volume of the pad. By averaging the results over ten samples ($5 \times 5 \text{ cm}^2$), the measurements yield a scaffold density of $0.024 \pm 0.001 \text{ g/cm}^3$. Since dry solid collagen has a density equal to 1.30 g/cm^3 , this yields a relative density $\frac{\rho^*}{\rho_s}$ of $1.86 \pm 0.05 \%$, which means that 1.86% of the volume is occupied, and 98.14% of the volume constitutes porosity content.

Through the 3D-analysis of the micro-CT scans of the collagen pad, the relative density, or percent object volume, was also computed and a value of 2.73% was obtained. As the collagen sample was examined at a $1 \mu\text{m}^3$ isotropic voxel size with micro-CT, fibers with a diameter below $1 \mu\text{m}$ are not detected and thus taken into account. Therefore, 2.73% is the relative density considering only the fibers which are thicker than $1 \mu\text{m}$. The percent object volume is calculated by CTAn software by taking the ratio of the volume occupied by binarised objects over the entire volume of the selected volume of interest.

Comparing both relative density values, they seem to be quite coherent, being of the same order of magnitude and not far from one another. However, they might be considered with a certain degree of uncertainty, as the first value (1.86%), averaged over ten samples, takes into account all fibers, yet yields a lower relative density than the micro-CT-generated value (2.73%), which only takes into account fibers with a diameter superior to $1 \mu\text{m}$.

Firstly, considering the micro-CT-generated value, one potential explanation is that the fibers with a diameter below $1 \mu\text{m}$ take up a small volume percentage relative to the thicker fibers which are detected. This is difficult to guarantee, but considering the scaffold as shown in Figure 3.1, it might be conceivable that such thin fibers do not significantly impact the occupied volume of the scaffold. Taking into account the thinner fibers might therefore realistically not yield a much higher relative density.

Furthermore, the value was computed based on the 3D-analysis performed, and therefore based on the chosen filters, threshold values, and some other morphological operations (cf. Section 2.2.4). The 3D-analysis parameters were chosen through

trial and error, based on 60 2D-scans among the entire set of scans, because of computational considerations. When the parameters (filter, threshold value, despeckling volumes) were chosen, they were then applied to the entire data set. The applied parameters were evaluated as correct as the visualization of the fibers seemed coherent, yielding full fibers and little noise. However, slightly different parameters could have yielded a somewhat different relative density. As the results with the chosen parameters seemed coherent, no other parameters were tested.

Another factor to consider is the number of samples used. Only one sample could be scanned with micro-CT, which means that it has to be considered as representative of the collagen sheets in general. Although this is probably the case, scanning a few more samples would provide a more representative value of the relative density. On the other hand, the value calculated by weighing and measuring the dimensions of the ten samples was averaged over ten samples, yielding a low-enough standard deviation for the mean value to be trusted.

In the end, it might not be possible to conclude on one exact relative density, but as both calculated values using very different methods have the same order of magnitude, it can be concluded that the pad contains a very high level of porosity, only a few percents (between 1 and 3 %) of its volume being occupied. Additionally, Varley *et al.* (2016) also calculated the relative density of a collagen scaffold produced through freeze-drying by weighing and measuring the dimensions with optical microscope. [13] They obtained a result of 0.5 % relative density, but based on SEM images concluded that 5 % occupied volume seemed more realistic. Similarly, Offedu *et al.* (2016) also measured the relative density of collagen scaffolds produced through freeze-drying and obtained values between 0.5 and 1 %. [34] In any case, these values also have a similar order of magnitude, which also helps to confirm that, in this case, the collagen pad has a relative density between 1 and 3 %, as obtained from both methods.

Table 3.2 provides an overview of the different relative density values computed in this thesis in comparison with the values from literature mentioned here above.

Table 3.2: Different relative density values from literature on other collagen scaffolds [13] [34] and computed in this thesis on HemopatchTM's collagen scaffold

	Method	Relative density [%]
Literature (other collagen scaffolds)	Optical microscope & balance	0.5 - 1
	SEM	5
Experimental (Hemopatch TM 's collagen scaffold)	Optical microscope & balance	1.86
	Micro-CT	2.73

3.2.2 Pore size distribution and fiber thickness distribution

3D-image analysis by micro-CT allows to measure a true, model-independent 3D-thickness and 3D-separation. The local thickness for a point in a solid is defined as the diameter of the largest sphere which fulfills two conditions:

- the sphere encloses the point,
- the sphere is entirely bounded with the solid surfaces.

Structure thickness CTAn calculates structure thickness by identifying the medial axes of all structures defined through binarisation, and then applying the “sphere-fitting” local thickness measurement as described here above for all the voxels lying along this axis. [58]

Structure separation It is defined as the thickness of the spaces established through binarisation. CTAn measures structure separation in the exact same way used for structure thickness as described here above, just applied to the space rather than the solid voxels. [58]

The structure thickness distribution and the structure separation distribution of the collagen pad measured from the micro-CT scans are shown in Figure 3.6.

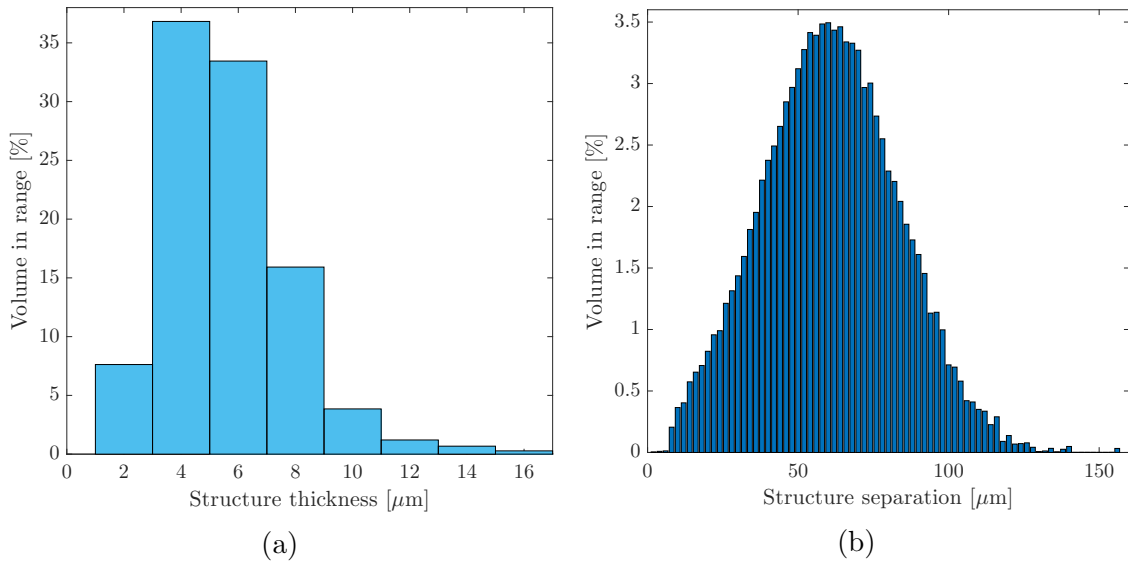


Figure 3.6: Structure separation and thickness distribution as measured with micro-CT 3D-analysis, taking into account only fibers with a diameter above $1\ \mu\text{m}$

The structure thickness distribution as presented in Figure 3.6a can be interpreted as the fiber thickness distribution of the collagen scaffold, but taking into account only the fibers with a diameter larger than $1\ \mu\text{m}$. Therefore, the real distribution should definitely show a higher proportion of fibers before the first bar of the histogram ($< 1\ \mu\text{m}$). Thus, considering only fibers thicker than $1\ \mu\text{m}$, 86 % of them have a

diameter between 3 and 9 μm . 8 % have a diameter between 1 and 3 μm and 6 % above 9 μm . These repartitions are summarized in Table 3.3. Only 0.17 % of the fibers have a structure thickness between 17 and 33 μm , but as this is such a small proportion, they are not shown in Figure 3.6a. This distribution yields an average fiber diameter of 5.61 μm .

Table 3.3: Structure thickness distribution obtained with micro-CT 3D-analysis, taking into account only fibers with a diameter above 1 μm

Structure thickness range [μm]	Volume in range [%]	Cumulative [%]
1 - 3	8	8
3 - 5	37	45
5 - 7	33	78
7 - 9	16	94
9 - 33	6	100

On the other hand, the structure separation distribution as shown in Figure 3.6b represents the pore size distribution, with an average of 60.88 μm . Since fibers thinner than 1 μm are not detected by the micro-CT, the distribution shown here is biased and shows many of the pores larger than what they are in reality. In fact, if the internal part of the scaffold has a similar architecture as what is shown in Figure 3.1, the pad has a non-negligible amount of thin fibers which lead to smaller pores than if just fibers thicker than 1 μm are considered. However, it is difficult to tell whether the distribution would in reality just be shifted towards the left, keeping the same distribution outline or whether the distribution would be flattened towards the left by a higher proportion of smaller pore sizes.

In the end, this shows how structure thickness and separation - or fiber thickness and pore size - are intrinsically and intricately related.

3.2.3 Pore size distribution and pore interconnectivity of pad as humid membrane

Particle size analysis is most often used to characterize particles in a suspension or emulsion, providing information about their size and shape. In this case, however, this analytical technique was used to characterize the collagen pad porosity, by filtering a suspension containing standard size particles through it and determining the cut-off size of the particles which would be allowed through or held back by the membrane. This would in turn provide an idea about the pore size distribution of the collagen pad, about the pore interconnectivity and potentially other information about its morphology. As the collagen pad is designed to function in a highly hydrated environment, filtering a suspension through it as a means to quantify its porosity and thus considering it as humid membrane is also relevant in this regard.

The first step was to analyze the standard size particles suspensions before filtering them through the collagen, as control group or reference. Two particle size analysis

instruments were used to do so. The concentrations of the suspensions used for both equipments were different and were chosen through trial and error to adapt to the detection capacity of each equipment. Afterwards, the goal was to filter the suspensions through the collagen pad and compare which particles were left in the suspension.

Using Parenteral Particle Counter (PPC), the suspension was successfully analyzed before and after the filtration through the collagen pad. The average of the three trials before filtration and the average of the three trials after filtration are shown in Figure 3.7. The software returns the number of particles per mL which have a diameter above 2 μm , above 5 μm , above 10 μm , above 20 and above 25 μm . In other words, the bars in Figure 3.7 represent the number of particles with a diameter

- between 2 and 5 μm ,
- between 5 and 10 μm ,
- between 10 and 20 μm ,
- between 20 and 25 μm ,
- above 25 μm .

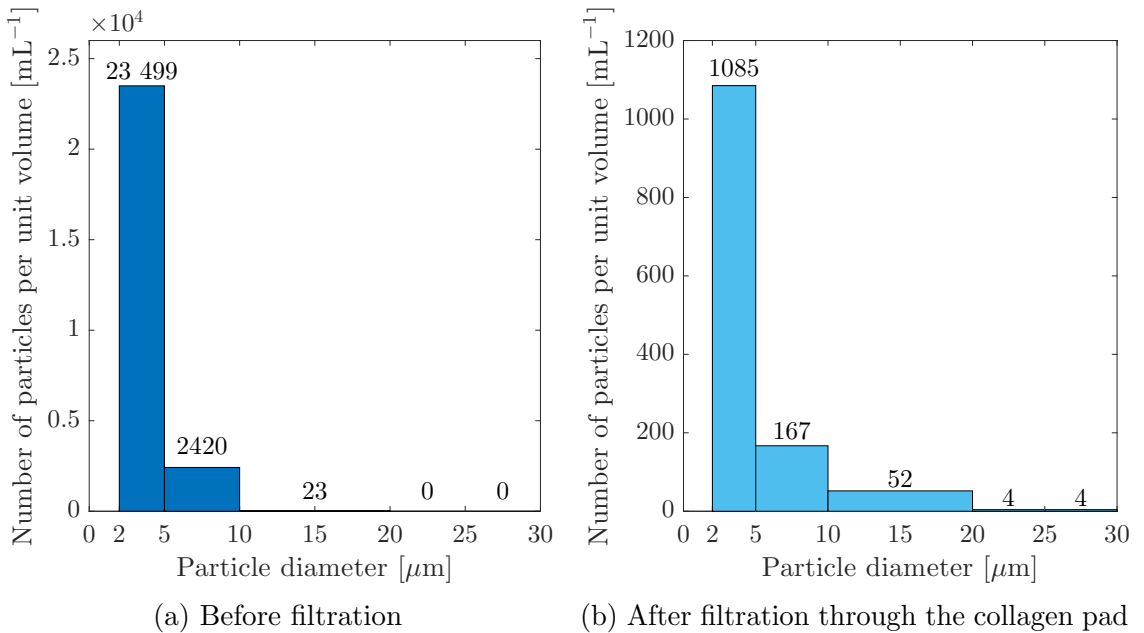


Figure 3.7: Particle size distribution obtained with PPC before and after filtration through the collagen pad

The number of particles with a diameter between 2 and 10 μm detected in the suspension after filtration (Figure 3.7b) has decreased by one order of magnitude compared to before filtration (Figure 3.7a), meaning that particles with a diameter between 2 and 10 μm are held back by the pad. However, many of the pores are larger than 10 μm , as seen in Figure 3.1. The fact that many of those particles are still held back by the pad is probably due to a quite low interconnectivity of the

pores, leading to only few paths which completely go through the pad. The many fibers with a diameter below $1\text{ }\mu\text{m}$ (Figure 3.1) do not represent a large volume compared to the larger fibers, and therefore probably do not contribute significantly to the pad's relative density. However, they most likely do play a role in holding back particles, thus decreasing the interconnectivity of the pores.

Another possible explanation for the significant decrease in the detection of 2-to-10- μm -diameter particles is linked to the function of the collagen matrix. As mentioned in Section 1.2.1, the collagen matrix plays an important role in HemopatchTM Sealing Hemostat in absorbing present fluids in the body. When passing 35 mL of the suspension through the collagen pad, 31 mL is obtained as filtrate, which means that 4 mL stays in the pad. With the 4 mL, a large part of the particles could stay stuck as well, due to the absorption capacity of the collagen scaffold.

On the other hand, another effect is observed for the particles with a diameter higher than $10\text{ }\mu\text{m}$. As indicated in Figure 3.7a, very little of the 10-to-20- μm -diameter particles present in the suspension before filtration were detected by the PPC (23 out of the theoretical 200 per mL). It would have been expected that an even lower number of particles would be detected in the suspension filtered through the collagen, meaning that particles would be held back by the pad. Instead of this, the number of detected particles with a diameter higher than $10\text{ }\mu\text{m}$ increases in the suspension after filtration (Figure 3.7b). This indicates that the collagen pad itself leaves behind residues when the suspension is filtered through it, residues which are quite large, even some larger than $25\text{ }\mu\text{m}$. One potential reason could be that the polystyrene particles were damaging the pad during the filtration, thus detaching fibers. However, the same large residues were detected when only Milli-Q water was passed through the collagen pad. This points more to a pad degradation induced by the filtration and water itself, rather than the PS particles.

Therefore, this in turn means that not all fibers in the collagen pad are held together, probably due to reasons linked to the fabrication process, such as the absence of crosslinking, fibers loosened during the freeze-drying process, or a poor homogenization of the collagen solution. Thus, this gives additional information about the collagen pad and its constitution.

Another equipment used was Occhio. The suspensions prepared for analysis with this equipment only contained particles with one standard diameter (2, 5 or $10\text{ }\mu\text{m}$) because the detection threshold could more easily be adjusted with only one size of particles. After correctly adjusting the threshold for each of the three suspensions so that enough particles would be detected for each of them, adequate distributions were obtained with Occhio as shown in Figure 3.8. Milli-Q water was also analyzed by itself and yielded a very low number of detected particles (on average, 5 per 0.3 mL analyzed).

When the suspension containing 2- μm -diameter particles was filtered through collagen and analyzed with Occhio, however, an unusually high number of particles was detected. After several trials, it was inferred that this was due to the release of larger particles by the collagen pad itself during the filtration of the suspension.

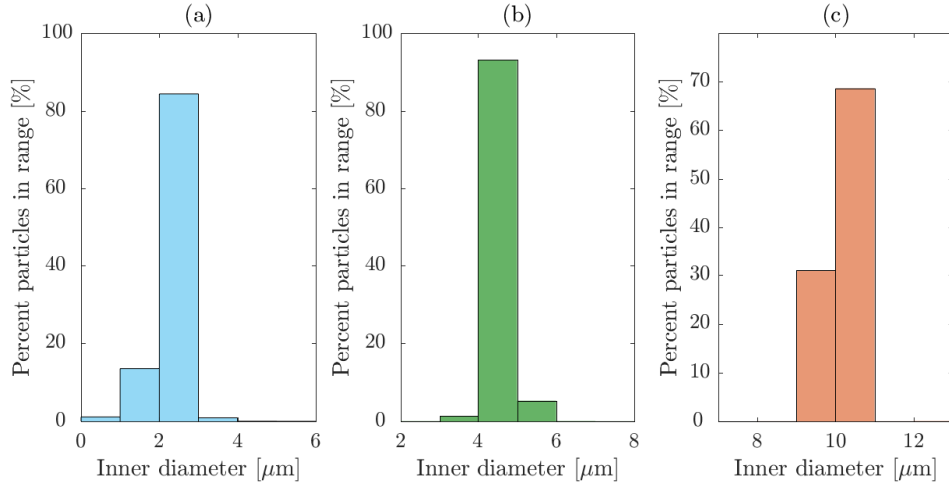


Figure 3.8: Particle size distribution obtained with Occhio for suspensions with standard size particles with a diameter of (a) 2 μm , (b) 5 μm , (c) 10 μm

In fact, it is possible to visualize the suspension analyzed by Occhio in real time, as well as the detected particles it has taken into account afterwards. Figure 3.9 shows 2- μm -diameter particles detected by Occhio, as well as much larger, non-spherical particles.¹ As the microcell through which the suspension goes in Occhio is only 50 μm thick, some of those large particles stay stuck or at least prevent the regular flow of the 2- μm -diameter particles, causing the same particles to be counted several times. Furthermore, the capillary going to the microcell is introduced in the cell with a 90° angle, which exacerbates this effect.

In the end, the detection of large and non-spherical particles with Occhio confirms that the increase in particles larger than 10 μm detected with PPC (Figure 3.7) in the suspension after filtration through the pad is indeed due to the release of residues by the collagen pad during the filtration.

Ultimately, particle size analysis combined with the visualization techniques of Section 3.1 allow to gain an insight into the scaffold architecture and its porosity, and particularly, it allows to infer that:

- most pores have a diameter larger than 10 μm ,
- the interconnectivity of the pores is quite small,
- the pad leaves behind residues, some of which have a diameter larger than 10 μm , just by the filtration and flushing of water through it, probably due the manufacturing process.

¹The red scale in the bottom of the image indicates 100 μm .

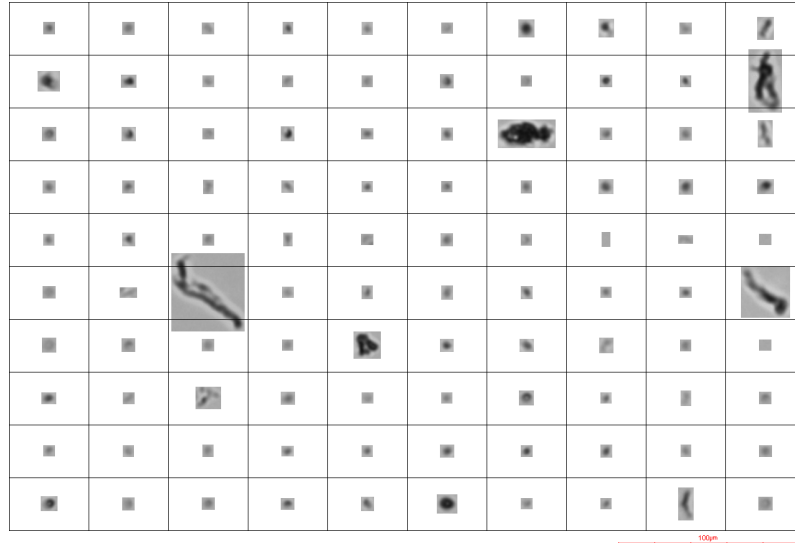


Figure 3.9: Particles detected by Occhio in the suspension filtered through the collagen pad. The small, spherical particles are standard $2\text{-}\mu\text{m}$ -diameter particles, while the other non-spherical particles are residues generated by the pad when water is filtered through it.

3.2.4 Degree of anisotropy

Isotropy is a 3D-measure of the preferential alignment of structures (or fibers in this case) along a particular direction. The collagen scaffold can be considered as a volume consisting of two phases: the fibers on the one hand, and the pores or space on the other hand. Therefore, the shape and size of the pores are necessarily linked to the fibers surrounding them, meaning that the scaffold's degree of anisotropy (DA) and porosity are intrinsically related. The degree of anisotropy was calculated from the micro-CT scans of the collagen pad using 3D-analysis.

CTAn software uses mean intercept length (MIL) and eigen analysis to calculate the DA. The volume of the scanned collagen pad is considered as containing two phases, solid and space. For the MIL analysis, a grid of lines is sent through the volume over a larger number of 3D-angles, as shown in Figure 3.10a, in which only two angles are illustrated. The length of each line going through the volume is divided by the number of times the line intercepts the solid phase. The MIL for each angle is calculated as the average of

$$\frac{\text{Length of line}}{\text{Number of intercepts with the solid phase}} \quad (3.1)$$

for all the lines of the grid. Thus, MIL with respect to a particular orientation is defined as the mean distance between a change from one phase to the other in such an orientation. [59]

Afterwards, all MIL lines are drawn passing through one point. An ellipsoid is fitted statistically to the 3D-distribution of MILs, and this ellipsoid has three axes or orthogonal vectors, as shown in Figure 3.10b.

These three vectors can be described as a 3x3 matrix, of which the three eigenvalues can be found. Finally, the DA is defined as:

$$DA = 1 - \frac{\lambda_{min}}{\lambda_{max}} \quad (3.2)$$

where λ_{min} and λ_{max} are the maximum and minimum eigenvalues. [58]

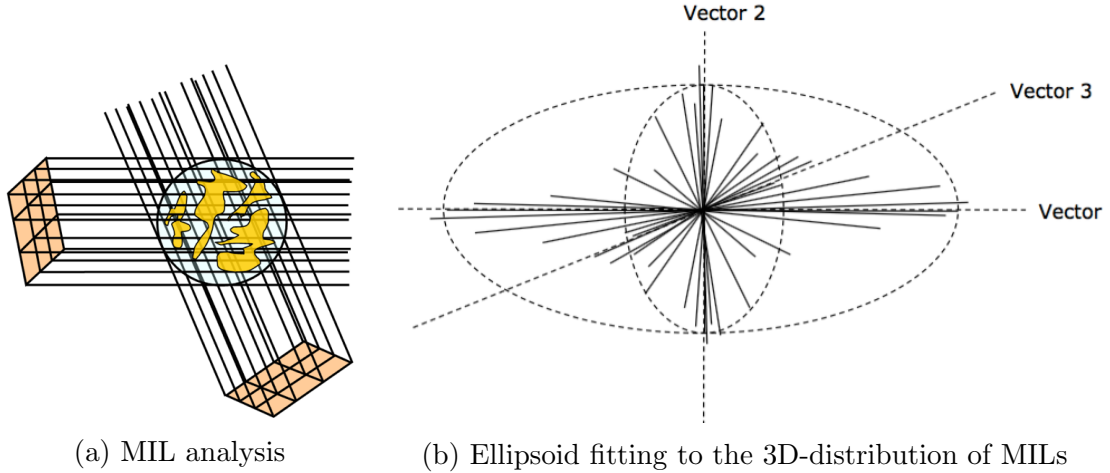


Figure 3.10: Degree of anisotropy (DA) calculation elements [58]

Thus, DA is 0 for a total isotropy and 1 for total anisotropy. For the imaged collagen pad, a DA of 0.33 was obtained. This means that the fibers do not form a perfectly isotropic structure but that the structure is more isotropic than anisotropic. This is again only considering fibers with a diameter above 1 μm .

As the collagen pad is not totally isotropic, this means that there must be one or several directions along which the fibers align preferably. VoxTex, a micro-CT data analysis software developed within KU Leuven, was therefore used to determine these preferential directions. Figure 3.11 shows the coordinate system used, in which the xy-plane is the projection plane. The x-, y- and z-directions shown in this coordinate system correspond to the x-, y- and z-directions used in the micro-CT 3D-reconstruction of the collagen pad in Figure 3.5. Furthermore, the xy-plane corresponds to the surface of the collagen sheet while the z-direction is the thickness of the sheet or sample.

The θ -orientation distribution is visible in Figure 3.12. As shown in Figure 3.11, it represents the angle between the z-direction and the xy-plane. The number of entries represents the number of fibers or other collagen components detected by the software and is 471244 in total. The θ -distribution displays a very wide peak - between 0 and 160° - meaning that, overall, the fibers do go in almost all directions. This is in line with the fact that the pad is more isotropic than anisotropic, as indicated by the DA. However, there is a maximum number of entries around $\theta = 90^\circ$. There is thus a preferential orientation of the fibers in the xy-plane, i.e. along or parallel to the collagen pad's surface, but with a remaining variation, due to the wide distribution. Comparing this with Figure 3.5, it seems coherent as the fibers seem to be oriented along the xy-plane in general, yet with some variation.

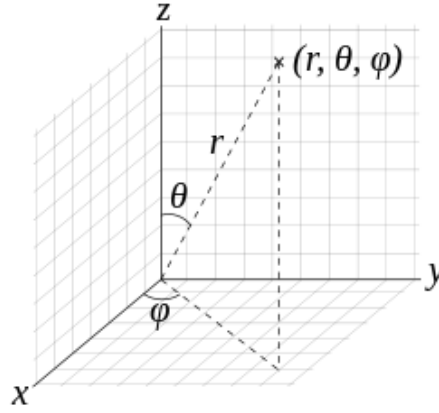


Figure 3.11: Spherical-coordinate system used as reference to analyze the fibers' preferential directions

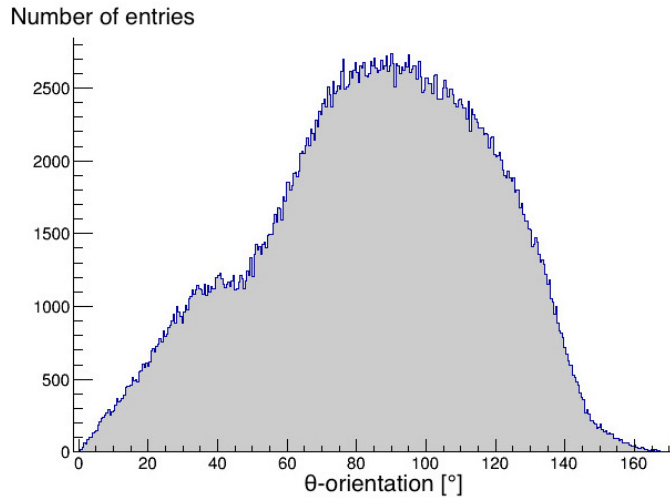


Figure 3.12: θ -orientation distribution of the collagen fibers, showing a preferential direction along the xy-plane but with a wide orientation variation remaining

Knowing that there is a preferential fiber alignment along the xy-plane, it is interesting to then identify whether there is a preferential direction of the fibers within this plane. This is shown by the ϕ -orientation distribution, which represents the angle between the x- and y-directions and is shown in Figure 3.13. Again, the total number of entries is 471244.

The ϕ -orientation distribution is very wide as well, but, unlike the θ -orientation distribution, it is bimodal. Two directions seem to be predominant: $\phi \sim 90^\circ$ and $\phi \sim -75^\circ$. This indicates that the fibers mostly follow the y- as opposed to the x-direction, but again with a somewhat large remaining variation in the orientation. Looking at the 3D-reconstruction of the pad in Figure 3.5, the preferential direction seems to match as most fibers seem to be oriented in the y-direction.

Before the micro-CT scanning of the collagen sample, careful attention was paid to which direction of scanning corresponds to the surface of the pad and which direction to its thickness. This is why it can be stated with certainty that the xy-plane is

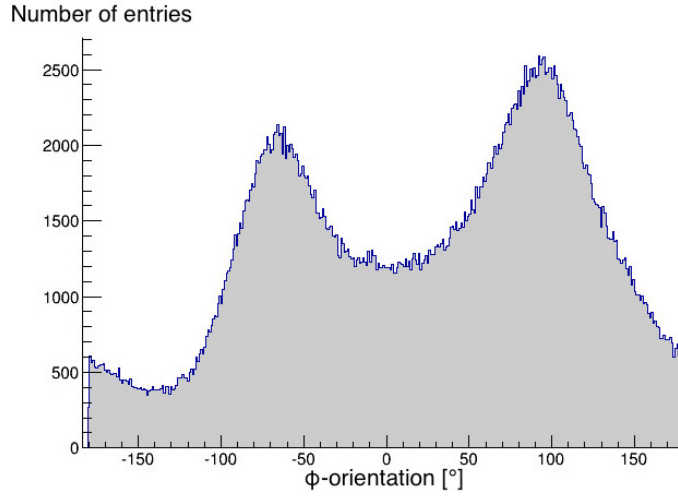


Figure 3.13: ϕ -orientation distribution of the collagen fibers, showing a bimodal preferential orientation of the collagen fibers along $\phi \sim 90^\circ$ and $\phi \sim -75^\circ$, but again with a large variation remaining

the pad's surface and the z-direction its thickness. However, no indication was made regarding the difference between the x- and y-directions during the sample preparation. In other words, it is not possible to assess whether the x-direction corresponds to the height or the width of the collagen sheet. Therefore, it is impossible to tell to which direction within the pad's surface the y-direction corresponds.

Ultimately, this additional 3D-analysis allows to gain an insight into the preferential orientation directions of the fibers. Again, it has to be kept in mind that only fibers with a diameter above $1 \mu\text{m}$ are taken into account. These fibers tend to primarily align in the xy-plane, thus along the pad's surface. Within this plane, there tends to be a preferential orientation in the y-direction. Unfortunately, it cannot be stated which direction this corresponds to within the pad's surface. Performing another micro-CT scan would allow to respond to this pending question. In fact, carefully noting during the sample preparation to which directions the x- and y- directions belong in the collagen surface plane would allow to identify the preferential fiber orientation within the collagen pad. In the end, despite the identified preferential direction, the collagen scaffold remains mostly isotropic with a DA of 0.33.

3.3 Thermal degradation

After the examination and quantification of the porosity of the unmodified collagen pad, the thermal degradation of the pad and its impact on the pad's structure was investigated. Gaining insight into the effect of heating on the pad could potentially provide a way to modify the pad's porosity in order to design a pad with a heat-controlled pore-size gradient, as explained in Section 1.4.

3.3.1 Shrinking of the pad

As it is known that the thermal degradation of a collagen scaffold leads to a macroscopic, visible shrinking, the first step was to visualize this degradation and its impact on the the collagen pad architecture.

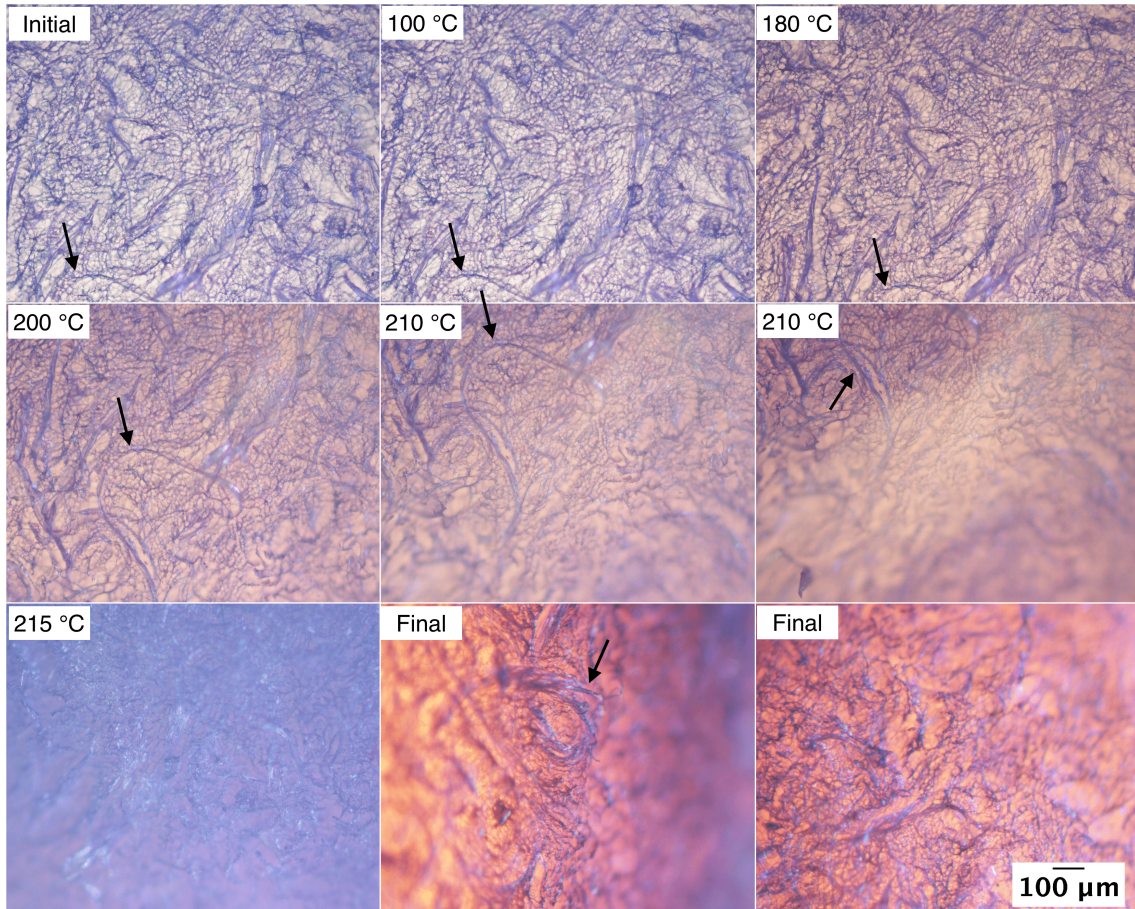


Figure 3.14: *In situ* heating of the collagen pad imaged with optical microscope, showing the pad at different temperatures and indicating one same fiber followed throughout the heating

In situ Using a heating plate, it was possible to image the collagen sample with optical microscope and SEM while heating it *in situ*. Figure 3.14 shows a collagen

sample imaged with optical microscope while heating it at 10 °C/min. The same location is imaged and a recognizable fiber is indicated with an arrow. From 25 up to 180 °C, no change is visible. Between 180 °C and 210 °C, the indicated fiber moves up, showing that the shrinking is occurring. At 215 °C, the pad has moved so much due to the shrinking that the same location cannot be found or recognized anymore. In addition, the shrinking causes it to be denser, allowing less light to go through, explaining why the sample looks darker. The two last images show the sample in its final degraded state (after heating up to 215 °C), after which no evolution is visible anymore. The sample is damaged and some fibers are sticking out of the sample, as indicated in the second-to-last image in Figure 3.14.

Both with SEM and optical microscope, using the heating plate to heat *in situ* presents some drawbacks. Firstly, the resolution of the imaging is definitely impacted and not as detailed features can be observed. Secondly, the shrinking of the pad inevitably leads to motion, making it difficult to follow one same location. Furthermore, the pad doesn't remain flat, making it impossible to obtain a focus on the entire image and causing the images to be blurry when fibers are out of the focus, as shown in Figure 3.14.

When using the heating plate with SEM, the vacuum is not as well preserved. Additionally, it was difficult to image the shrinking scaffold with SEM and to resolve structural differences while heating. Because of these drawbacks, it was chosen to only show the *in situ* heating images obtained with optical microscope (Figure 3.14).

Ex situ Because of the artefacts generated by *in situ* heating, *ex situ* heating was turned to. The pad was heated in an oven at different temperatures for various times and the generated effects on the collagen scaffold were observed. At this stage, the pad degradation was evaluated macroscopically, based on two criteria: its dimensions and the color it turned to. The aim was to find a degradation state at which the pad had shrunk the most, without burning (which was defined as the sample turning brown), in order to perform the analyses presented in the following sections on one same degradation state. Several heating times and temperatures were tried, and it was decided that the sample reached such a state without burning when the sample was heated at 225 °C for 4 minutes in the oven. The unmodified collagen pad, an intermediate state pad, heated at 200 °C for 20 minutes and the pad heated at 225 °C for 4 minutes in the oven are shown macroscopically in Figure 3.15 and their dimensions in Table 3.4.

After evaluating the effects of heating macroscopically, the consequences on the porosity were first visually analyzed with SEM. Figure 3.16 shows three different collagen pads imaged with SEM *ex situ* at three different degradation stages: before heating, after heating at 200 °C for 20 minutes (intermediate state) and after heating at 225 °C for 4 minutes (defined as the final or most degraded state before burning).²

²The scale applies to all three images.

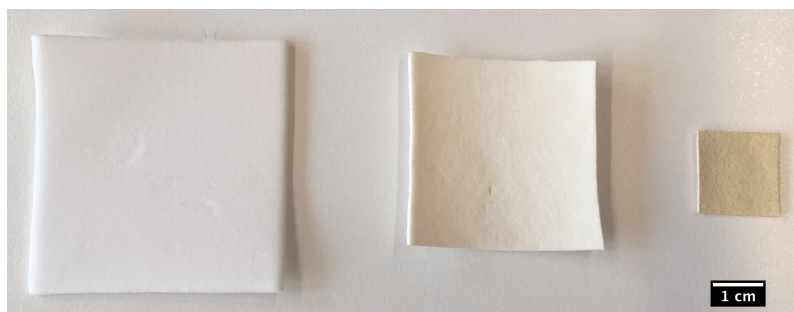


Figure 3.15: Undegraded collagen sample (left), degraded sample after heating at 200 °C for 20 minutes (middle) and degraded sample after heating at 225 °C for 4 minutes in an oven (right)

Table 3.4: Dimensions of the unmodified sample, a degraded sample after heating at 200 °C for 20 minutes and a degraded sample after heating at 225 °C for 4 minutes in an oven

	Unmodified sample	Degraded sample after heating at 220 °C for 20 minutes	Degraded sample after heating at 225 °C for 4 minutes
Surface dimensions [mm ²]	~ 50 x 50	~ 27 x 27	~ 16 x 16
Thickness [mm]	~ 2.10	~ 1.20	~ 0.75

As displayed in Figure 3.16, the pore size decreases significantly between the unmodified, intermediate and final stage samples. In the final stage, the sample has collapsed onto itself and the shrinking causes some fibers to rise out of the sample, as observed *in situ* in Figure 3.14.

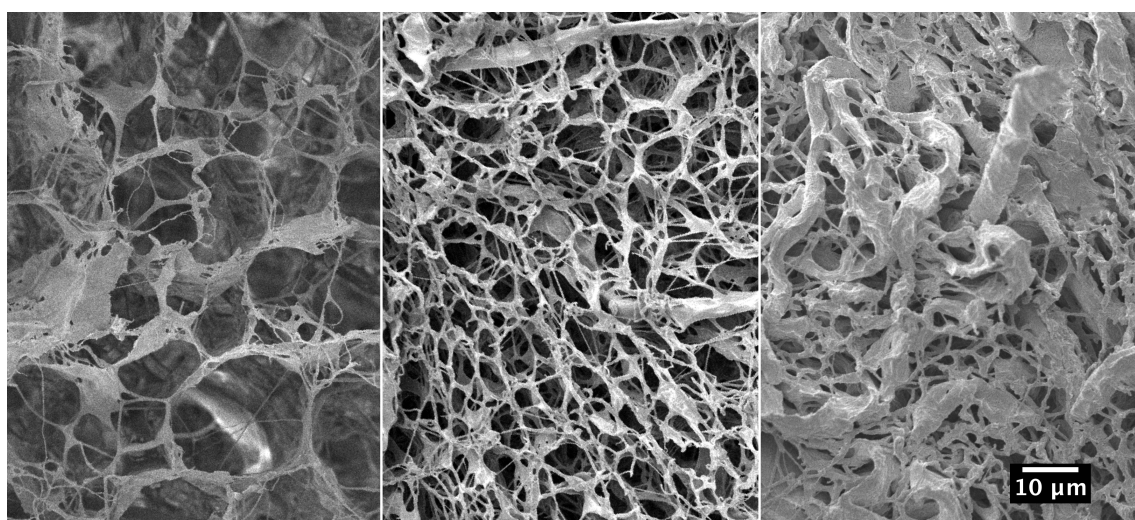
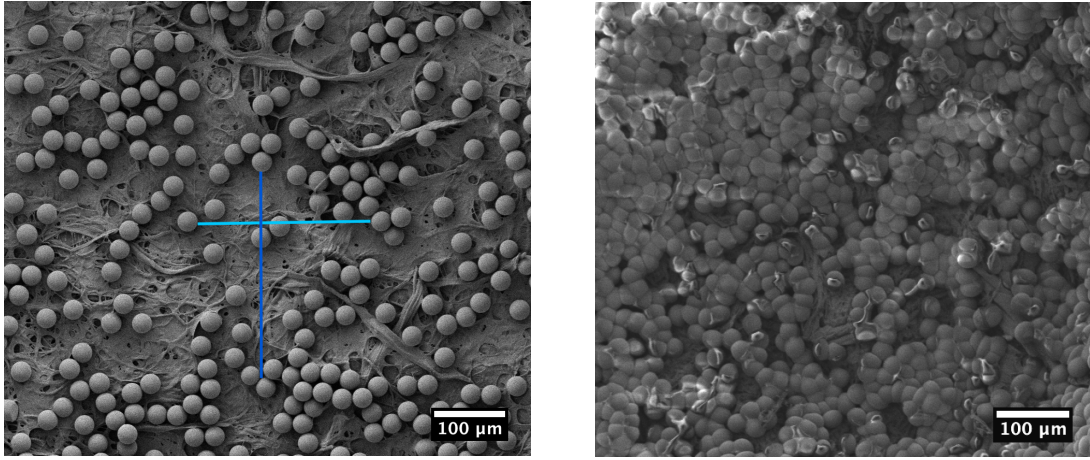


Figure 3.16: Collagen pad unmodified (left), heated at 200 °C for 20 minutes (middle) and heated at 225 °C for 4 minutes (right), imaged with SEM

Isotropic shrinking As heating the pad causes it to shrink, the question of whether the shrinking occurs isotropically along the pad surface (x- and y-directions) arises. As explained in more details in Section 2.5.1 and shown in Figure 2.4, a suspension with standard size PS particles was dropped in specific spots of a collagen sample. As shown in Figure 3.17a, the horizontal and vertical distances between chosen particles were measured in two different areas of the pad (only one is shown). To measure the evolution of the shrinking in both directions, the sample was gradually heated and the same distances were measured after each heating step. Figure 3.17a shows the initial distances before heating the sample.



(a) Dimension measurements in both directions on unmodified sample

(b) Sample with standard size particles after heating at 225 °C for 1 minute, preventing the distances to further be measured

Figure 3.17: Distance measurements between standard size particles on the collagen pad for determining whether the shrinking of the pad occurs isotropically

Figure 3.18 shows the relative distances (compared to the initial distance) as a function of the temperature at which the sample had been heated before imaging. The relative distances between the particles in Figure 3.18 do not go until maximum shrinking of the pad, because at that point, the particles were on top of each other, as shown in Figure 3.17b, and the distances could not be measured anymore. This occurred after the last heating step as described in Section 2.5.1, when heating the sample at 225 °C for 1 minute.

In both Figures 3.18a and 3.18b, the distances between the particles in the horizontal and vertical directions decrease to very similar extents at each temperature. The highest deviation observed between the evolution of the distances in x- and y-directions is 3 %. In the end, this quantitatively shows that the shrinking does occur isotropically. In Section 3.2.4, it was determined through micro-CT 3D-analysis that the undegraded sample is quite isotropic, based only on fibers with a diameter larger than 1 μm . Connecting both results together, it is interesting to find that this isotropic structure leads to an isotropic shrinking when heated.

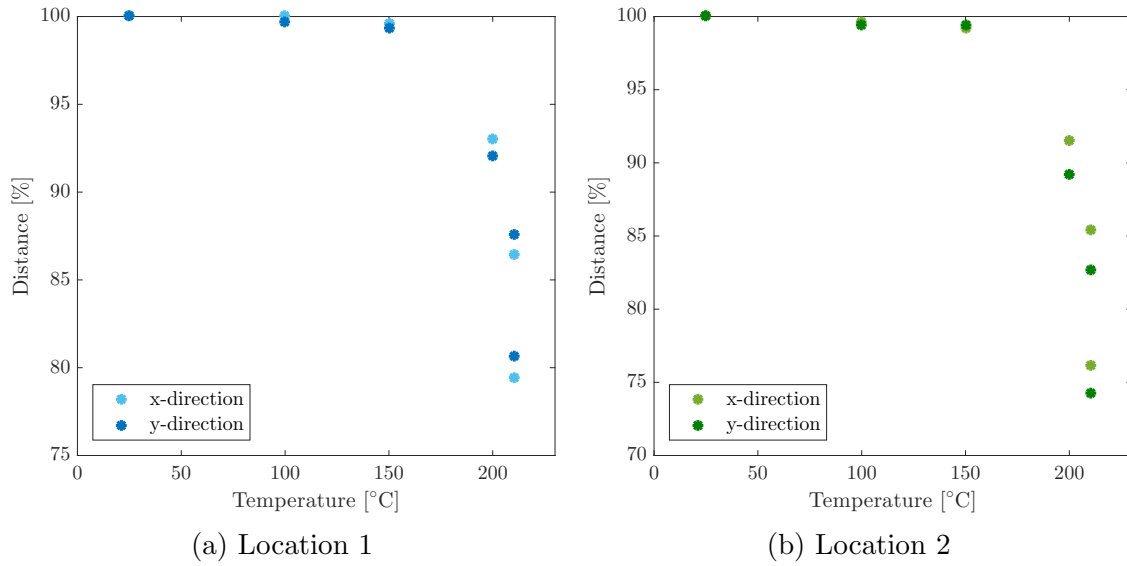


Figure 3.18: Horizontal and vertical tracking of distances in two locations on the collagen pad with SEM as a function of temperature, showing the shrinking occurs isotropically

3.3.2 Relative density after degradation

The relative density $\frac{\rho^*}{\rho_s}$ (Equation 1.1) of the collagen pad after heating and thermal degradation was also measured. The same ten samples were used as in Section 3.2.1, heated at 225 °C for 4 minutes in an oven because this was evaluated as being a limit state before the sample burns, as explained in Section 3.3.1. The resulting average mass, surface area and thickness of the shrunk samples compared with the non-degraded samples are displayed in Table 3.5. This leads to a scaffold density of 0.597 ± 0.026 g/cm³ and a relative density $\frac{\rho^*}{\rho_s}$ of 45.92 ± 2.01 %.

Table 3.5: Comparative table of the relative density of the unmodified and thermally degraded collagen samples

	Unmodified collagen	Collagen after heating (225 °C for 4 min)
Mass [g]	0.127	0.112
Surface area [cm ²]	25.288	2.513
Thickness [cm]	0.210	0.075
Density [g/cm ³]	0.024 ± 0.001	0.597 ± 0.026
Relative density [%]	1.86 ± 0.05	45.92 ± 2.01

Ultimately, this significant increase in relative density seems coherent with the change in porosity observed in Figure 3.16, showing a much denser scaffold after degradation.

As mentioned in Section 1.3, collagen scaffolds are often considered as 3D-cellular solids, consisting of pores with no walls delimited by struts of material forming the edges or faces of cells. [32] In addition, collagen scaffolds have also been shown to have similar mechanical properties to open-cell foams.

In fact, from cellular mechanics, the stiffness E of such porous collagen scaffolds is expected to be directly proportional to the square of their relative density:

$$E \propto \left(\frac{\rho^*}{\rho_s}\right)^2 \quad (3.3)$$

No mechanical testing was done in this thesis, but using the relative densities of the unmodified and degraded collagen scaffolds, it is possible to predict the change in stiffness caused by the increase in relative density with heating.

In fact, as the relative density was found to increase from 1.86 to 45.92 % (Table 3.5), stiffness would increase by three orders of magnitude:

$$E_{\text{unmodified}} \propto (1.86)^2 \propto 3.5 \quad (3.4)$$

$$E_{\text{degraded}} \propto (45.92)^2 \propto 2108.6 \quad (3.5)$$

Therefore, heating the pad up to when its volume decreases by a factor of 2 would increase its stiffness four-fold, assuming no change in mass occurs. In this case, the collagen pad is definitely stiffer after heating, and according to this cellular mechanics model, the collagen becomes almost three orders of magnitude stiffer after heating at 225 °C for 4 minutes. Putting this into perspective, designing a pad with a pore-size gradient would not only provide different hemostatic and permeability properties along the gradient, but it would also allow to modulate the pad's mechanical properties and provide various stiffnesses.

3.3.3 Pore size distribution and pore interconnectivity of degraded pad as humid membrane

PPC was used again to assess whether the cut-off size of particles able to pass through the degraded pad would be different compared to the initial, unmodified pad. The same suspension as the one used in Section 3.2.3, containing 2-, 5-, 10-, and 20- μm -diameter particles, was passed through three samples, which this time had been degraded through heating at 225 °C for 4 minutes (again same degradation state). Figure 3.19 shows the comparison between the number of particles per mL gone through the unmodified and the degraded samples.

Compared to the unmodified scaffold, less particles with a diameter between 2 and 10 μm are able to go through, as indicated by the first two bars of Figure 3.19b compared with Figure 3.19a, although more than half are not held back by the pad. The pad used for this experiment has been degraded to the same extent as the one shown on the right in Figure 3.16 and in Figure 3.20b. Comparing the number of particles detected with the SEM-imaged scaffold in Figure 3.20b, it seems coherent that a significant proportion of the 2- and 5- μm -diameter particles are still able to go through. In fact, the pad has shrunk compared to the initial pad (Figure 3.20a),

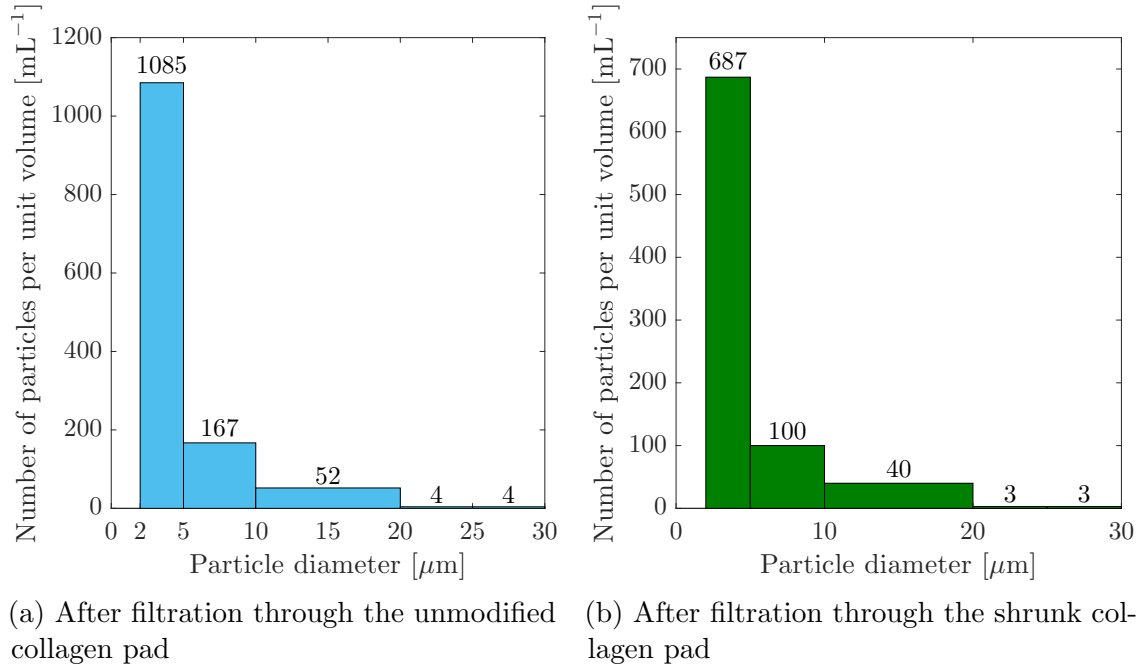


Figure 3.19: Particle size distribution obtained with PPC after filtration through unmodified and shrunk collagen pads

leading to smaller pores, but most pores seem to have maintained a diameter larger than $5\ \mu\text{m}$. While the SEM image only gives information about a small part of the surface of the pad, the particles detected by the PPC provide a quantification of the size of the pores in the middle of the pad, not accessible with SEM. This technique in fact confirms that for this degradation state, the scaffold retains pores primarily with a diameter larger than $5\ \mu\text{m}$ even within the bulk of the pad.

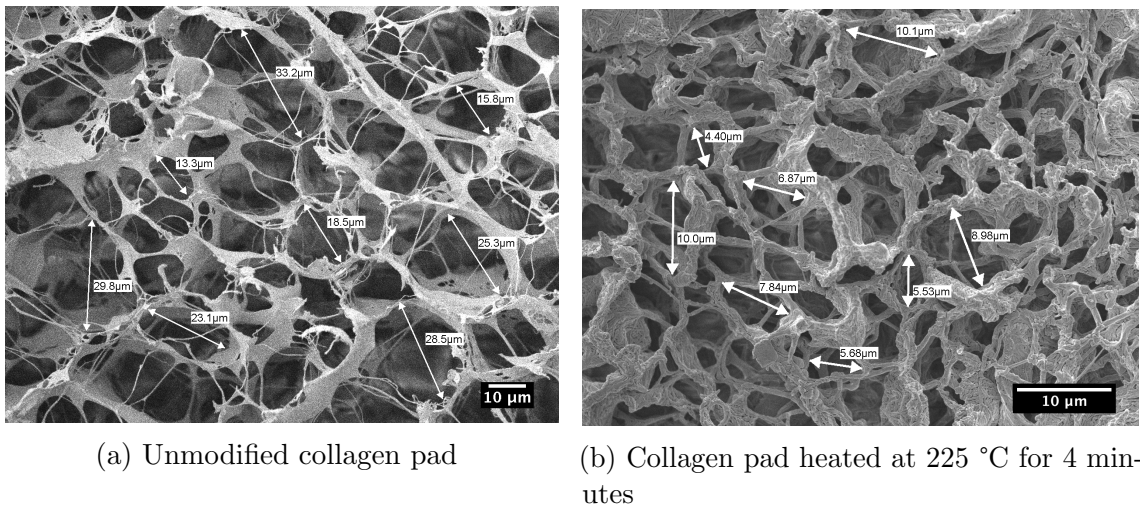


Figure 3.20: Collagen scaffolds imaged with SEM, showing a few pore diameter measures

As to the particles with a diameter above $10\text{ }\mu\text{m}$, the same observation is made as for the suspension which went through the unmodified collagen: looking at Figure 3.20b, these particles cannot go through the scaffold, but instead, the pad itself leaves residues behind. The slightly lower number of those particles compared to the case of the unmodified collagen can be due to the fact that the pores are now smaller, so that less of those fiber debris can go through the pad itself.

In the case of the degraded sample, however, Occhio was not used to analyze the suspension filtered through it, because it did not allow to accurately analyze the suspension, as explained in Section 3.2.3. Therefore, the size of the particles is known but not their shape. No certainty is therefore ensured on the origin or nature of those large particles. However, as the distribution for the degraded pad (Figure 3.19b) is similar to the one for the unmodified pad (Figure 3.19a), it can be inferred that these large particles are residues as well.

In the end, particle size analysis allows to compare the porosity of the original sample with the degraded sample and provides the following information about the comparison between both scaffolds:

- the degraded sample's pores have shrunk compared to the original sample, but still mostly remain with a diameter superior to $5\text{ }\mu\text{m}$,
- the interconnectivity of the degraded sample remains low,
- the degraded pad also leaves behind residues, among which again some with a diameter above $10\text{ }\mu\text{m}$.

3.3.4 Possibility to partially degrade the pad

As shown in Figure 3.16, thermal degradation of the collagen pad leads to a decreased pore size. This introduces the possibility of using heating as a means to control the pore size of the collagen scaffold. As a first investigation of this, an aluminum grid was heated to reach $300\text{ }^{\circ}\text{C}$ in an oven and placed on top of a collagen sample. This allowed to degrade only the part of the pad in contact with the grid, while the other parts - the inside of the circles - remained intact, as shown in Figure 3.21a.

Figure 3.21b shows the limit between the degraded and non-degraded zone imaged with SEM, while Figures 3.21c and 3.21d show each zone separately at a higher magnification. By heating the grid at $300\text{ }^{\circ}\text{C}$, the pad was not degraded up to its final or maximum state (as opposed to Figure 3.16 on the right) because the grid cools down quite rapidly, not being able to shrink the sample as much, but despite this, a clear difference is noticeable in the porosity and the state of the fibers in both zones. The difference in architecture is not due to the pressure of the grid on the pad only, because, when the cold grid is applied on the pad, the latter is in that case completely able to recover its original state. Thus, the difference is truly due to the degradation of the collagen with heat, not due to the physical pressure of the grid.

In this case, no local gradient of pores was obtained but, using another technique, this could be further investigated and such a gradient could be obtained on a smaller

scale. This experiment thus demonstrates that degrading the pad more locally is possible and that the differentiation between the degraded and non-degraded states of the pad could provide a way to generate a pad with zones of different pore sizes. Furthermore, as discussed in Section 3.3.2, the shrinking leads to a higher relative density and an increase of stiffness in consequence. Thus, in this particular example, the degraded zones would not only have smaller pores, but also be stiffer, which could prove to be interesting for a future application or development of collagen scaffolds.

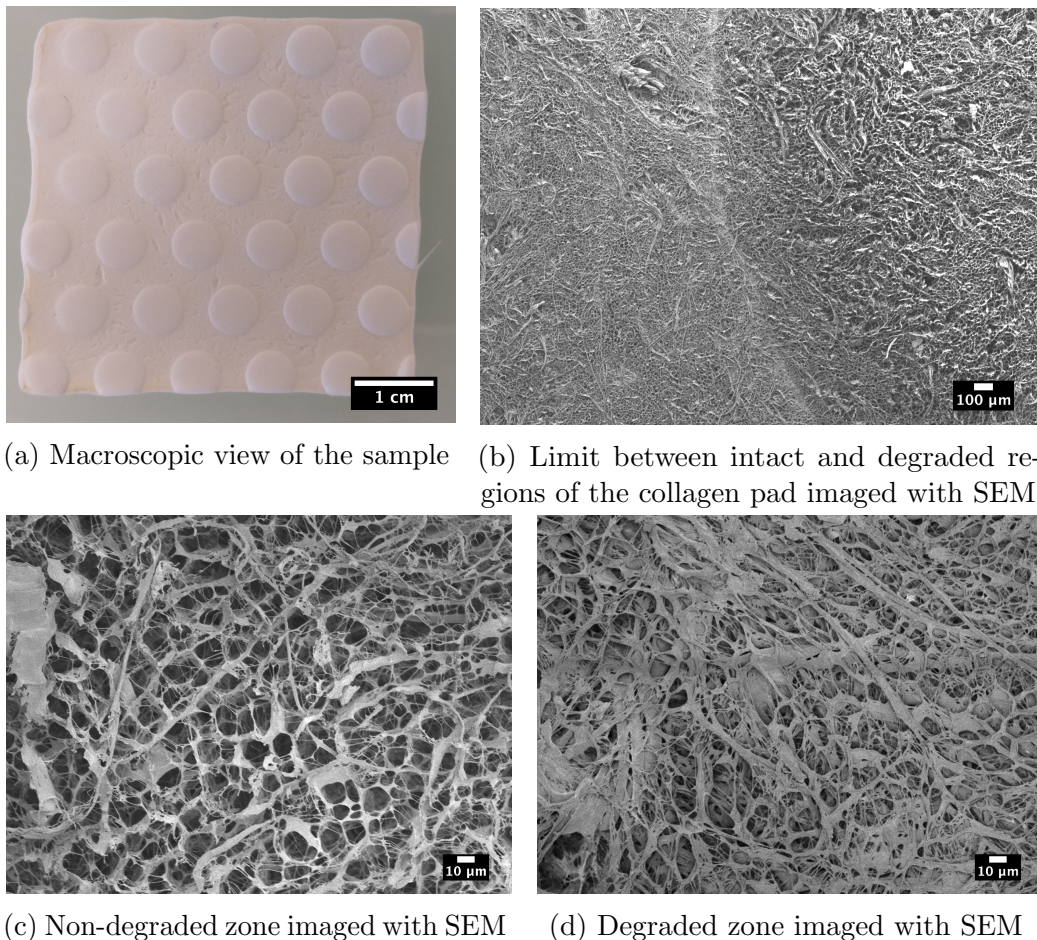


Figure 3.21: Collagen pad partially degraded using a pre-heated grid

3.3.5 Thermal transitions identification through DSC

As explained in Section 1.4.2, DSC has become a standard bulk thermoanalytical technique to study collagen denaturation, as the latter generates an endothermic peak. In literature, most collagen scaffolds are reported to have a denaturation temperature below or at most at 100 °C. [45] [49] [60] However, it was observed in earlier heating trials mentioned in the previous sections that the shrinking or physical degradation of this collagen pad is visible only from 180 °C and up when heating *in situ* as shown in Figure 3.14.

It was therefore not interesting to heat up only to 100 °C. This is why the pad was analyzed with DSC in a cycle from 5 up to 300 °C, with a heating rate of 5 °C/min. A pad heated at 225 °C for 4 minutes beforehand in an oven was also analyzed with the same heating conditions in DSC, and both curves are shown in Figure 3.22. Both were normalized according to the mass of the sample.

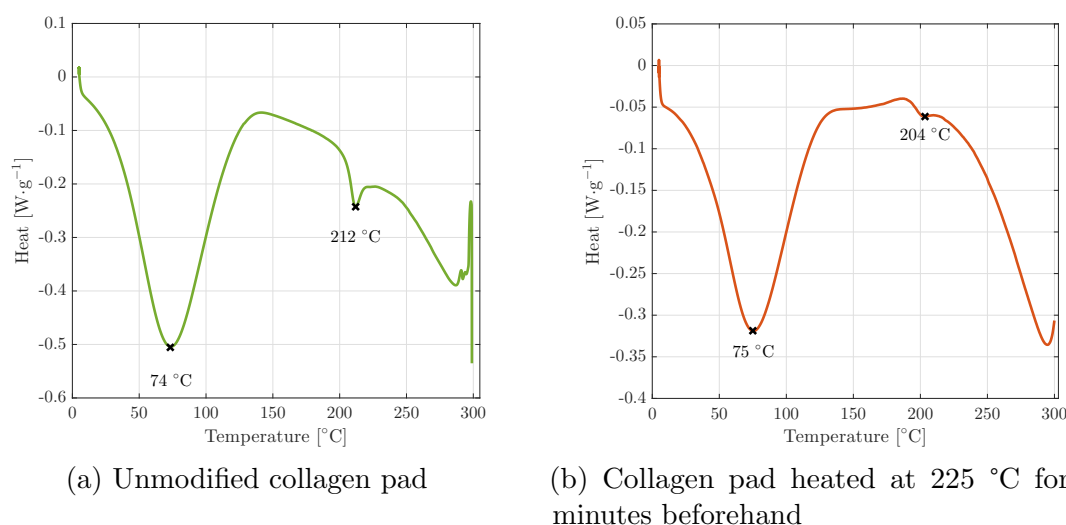


Figure 3.22: Heat flow as a function of temperature of unmodified and modified collagen pads, measured with DSC, with a heating rate of 5 °C/min

Unmodified pad An endothermic peak is observed at 74 °C (Figure 3.22a), corresponding to what is called the collagen *denaturation* peak in literature, as it is a large peak and usually occurs around this temperature range. It corresponds to the breaking of the hydrogen-bonds which stabilize and hold together the collagen triple helix, as explained in Section 1.4.1. [42] A second peak is visible at 212 °C, temperature which correlates with the temperature at which degradation and shrinking are observed *in situ* and *ex situ*. After 270 °C, some more deviations are present but at that point, the pad has completely burned.

Degraded pad As to the heat curve of the pre-degraded pad in Figure 3.22b, the same endothermic peak as for the unmodified pad is visible at 75 °C. Assuming this peak has the same origin as for the unmodified collagen pad, since the temperature range is the same, this indicates again the breaking of the heat-labile H-bonds of the collagen triple helix. As a reminder, the heating of the pad at 225 °C for 4 minutes (before the DSC analysis) degrades the pad such that its relative density increases from 1.86 % to 45.92 % (Section 3.3.2), making it physically collapse with smaller pores (Figure 3.16) and more rigid. However, despite these significant structural changes, it appears that, on a molecular level, the collagen triple helix H-bonds are still present and broken during the DSC-analysis at 75 °C. Therefore, this suggests a quite essential difference between the structural, visible thermal degradation of the pad and the molecular *denaturation* of the pad.

One potential explanation for the presence of this endothermic peak even though the pad has already shrunk with the heating in the oven is the following: it is possible that by heating the sample at 225 °C for 4 minutes, the heating time was not sufficient for the pad to completely denature. This means that not all the H-bonds of the entire mass of the sample were broken during the 4 minutes, and some of them are only broken during the heating with DSC.

The second peak present at 212 °C in Figure 3.22a is barely visible for the pre-degraded pad in Figure 3.22b. The endothermic character of the curve at 204 °C is so small that it can be questioned whether it in fact represents a true change. The same behavior was observed for the two other samples with the same pre-treatment and DSC analysis, indicating that this is not just an irregularity in this particular sample's curve. Again, this peak could be the remainder of the shrinking which did not fully complete during the 4 minutes in the oven. This would also explain why it is much smaller than for the unmodified pad in Figure 3.22a.

Reversibility As mentioned in Section 1.4.1, researchers still disagree whether collagen thermal denaturation is reversible [44] or irreversible [40] [45]. In order to investigate whether the denaturation peak at ~ 75 °C, as well as the second peak present at 212 and 204 °C in Figures 3.22a and 3.22b respectively are reversible, another DSC heating method was applied to both the unmodified and pre-degraded samples. The heating rate was kept at 5 °C/min.

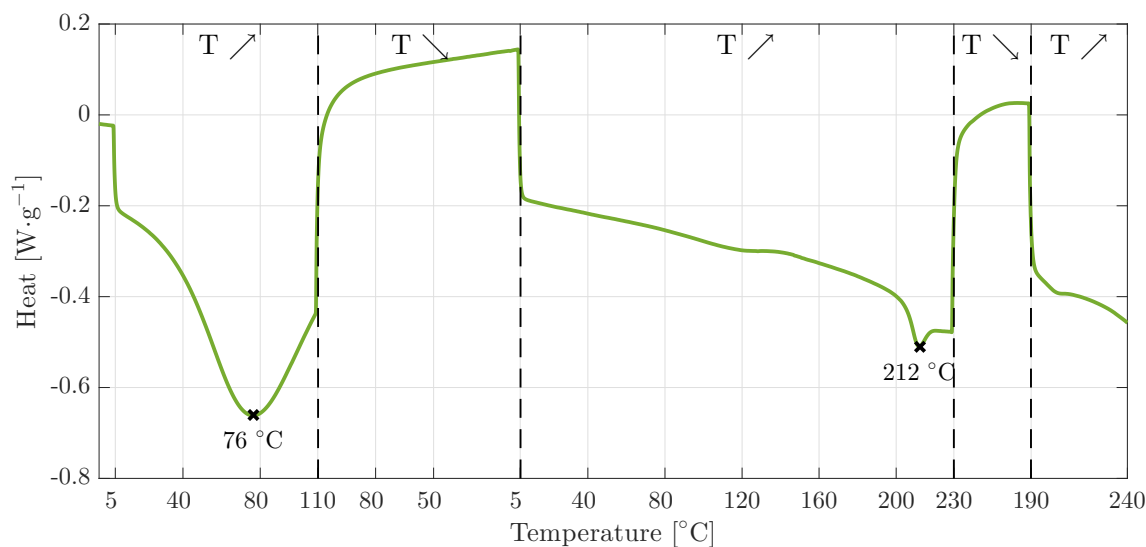


Figure 3.23: Heat flow as a function of temperature of collagen pad, with alternating heating and cooling ramps (5 °C/min), measured with DSC

First, the samples were heated from 5 up to 110 °C, cooled back down to 5 °C, and heated up again. This aimed at identifying whether the denaturation peak is present again during the second heating. Both curves in Figures 3.23 and 3.24 show a first endothermic peak at 76 °C, but nothing while cooling back down, and this peak is not present when heating the second time. Therefore, this indicates that this peak

- corresponding to the heat-breaking of the collagen triple helix H-bonds - is not reversible.

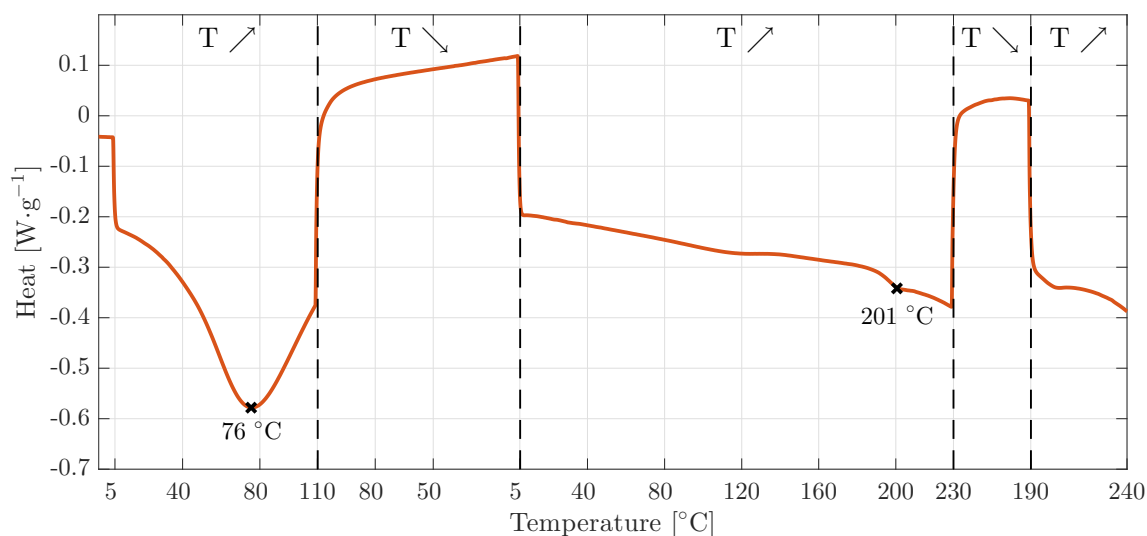


Figure 3.24: Heat flow as a function of temperature of degraded pad beforehand, with alternating heating and cooling ramps (5 °C/min), measured with DSC

During the second heating, the samples were heated up to 230 °C. Again, the unmodified sample's heat flow shows a second endothermic peak at 212 °C (Figure 3.23), while the already-degraded sample again has a very small endothermic peak at 201 °C (Figure 3.24). The samples were then cooled back down to 190 °C, and heated back up to 240 °C, to see whether the second endotherm would be again present. This proves not to be the case, meaning that the second endothermic peak is not reversible either.

Synopsis In the end, the collagen pad is denatured on the molecular level at ~ 75 °C, but this does not correspond to a visible structural damaging of the sample. However, a second endothermic peak at ~ 212 °C correlates with the temperature range in which the sample significantly shrinks. At this stage, the exact nature of this peak has not been identified, though. Both peaks refer to an irreversible denaturation and degradation respectively, as both are not present during a second heating.

Regarding the sample heated in the oven at 225 °C for 4 minutes beforehand, it is clear that this heating structurally degrades the sample, as it shrinks and reaches a volume one order of magnitude lower than originally. Nonetheless, it appears that this treatment has not completely denatured the sample on a molecular level, as the denaturation endotherm is still visible on the heat flow curve of these samples. This is possibly due to the limited heating time, which might not have allowed to fully denature the sample, leaving some hydrogen bonds which still break during the DSC first heating ramp. In fact, during the second heating of this sample, the denaturation peak at ~ 75 °C is not present, meaning that the sample has been completely denatured during the previous heating ramp.

In order to justify this, another experiment could be attempted. The sample would first be heated in the oven at 225 °C, but for a longer time, such as 10 minutes, and then analyzed with DSC. The first heating with DSC might then show a heat flow with no denaturation endotherm (~ 75 °C), confirming that, in order to have a complete denaturation, the heating has to be long enough for all the H-bonds to break.

On the other hand, the peak at ~ 212 °C is barely present for the pre-degraded sample, showing the role of the heating time not only in the degradation, but also the irreversible character of the degradation it corresponds to, since it is not present during the second DSC heating.

Literature Even though most scientific literature addressing collagen thermal degradation or denaturation presents results only up to ~ 100 °C, a few papers do mention results above this temperature. Guan *et al.* (2006) used DSC to assess the thermal properties of a collagen scaffold up to 250 °C. They found a broad endothermic peak between 52 and 170 °C and a second endothermic peak at 222.5 °C. [61] Similarly, Samouillan *et al.* (2003) heated different prepared collagen scaffolds up to 250 °C. They obtained a broad endothermic peak between 50 and 150 °C, and a second, narrower peak at ~ 225 °C. [62] Even though the first endothermic peak in both cases is much broader than what was obtained for HemopatchTM's collagen pad, the second peak is very similar: narrower and occurs at a similar temperature range. Although no explanation is given for the origin of the peaks, it is relevant to note that the second endothermic peak has also been observed for other collagen scaffolds.

Additionally, Sarti *et al.* (1995) used DSC on collagen samples, heating from -80 to 180 °C, quenching to -80 °C, and heating back to 180 °C. They observed a first peak at ~ 48 °C and a second one at 117 °C during the first heating ramp. During the second heating ramp, neither of those peaks is present anymore, and it is concluded that the sample has been degraded and has lost its organized structure. [63] Despite the difference in the temperatures at which the peaks arise compared to the collagen pad of this thesis, and despite the lack of specific interpretation of those peaks, it is relevant to note that irreversible peaks are observed in this case as well.

3.3.6 Thermo-mechanical behavior evolution of the pad

With the collagen pad's thermal degradation comes the question of how its mechanical properties evolve with temperature. The storage and loss shear moduli of the collagen pad were measured while heating the pad, by applying a shear-mode oscillatory stress onto the sample and recording the resulting strain in the sample's elastic domain. The results are shown in Figure 3.25.

The storage modulus G' , a measure of the sample's elastic behavior, is constant up to ~ 210 °C. At this temperature, the storage modulus starts to drop, which is often associated with the flow of a material. On the other hand, the loss modulus G'' , a

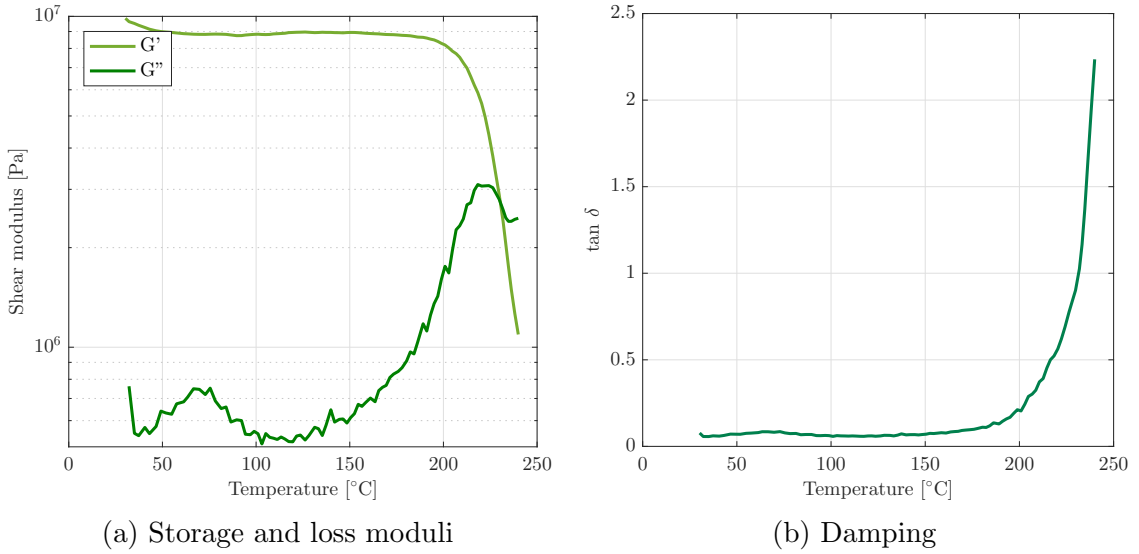


Figure 3.25: Evolution of the storage modulus G' , loss modulus G'' and damping $\tan \delta$ of HemopatchTM's collagen pad with temperature

measure of the viscous response of the material and thus of losses in the form of heat or friction, reaches a maximum at 225 °C.

$\tan \delta$ is the ratio of the loss modulus to the storage modulus, and is often called damping. It's a measure of how a material dissipates or absorbs energy. [64] At 200 °C, $\tan \delta$ significantly increases, which again is often associated with the flow of the material.

Thus, all three curves seem to indicate that the collagen pad starts to flow at ~ 200 - 210 °C, which is certainly not the case. In fact, this temperature corresponds to the temperature at which the second endothermic peak occurs with DSC (Figure 3.22a), and at which the sample significantly shrinks. Yet, after shrinking at ~ 210 °C, the pad is denser and stiffer. Therefore, it does not make sense to have flowing of the sample in this temperature range.

It is important to note that the shear modulus evolution, and thus of $\tan \delta$ as well, depends on the geometry of the sample. This introduces an error on two levels. Firstly, in order to be able to apply a stress in shear mode, the sample has to be held quite tightly. Since the sample is soft and flexible, it is therefore compressed. So the original geometry taken into account by the DMA and used for the moduli calculation is already approximated from the start, making the value of the storage modulus questionable. Secondly, the sample's geometry is considered constant during the measurement, which it is not in reality, since the pad shrinks with temperature. This is therefore a significant limit of the use of DMA for the collagen pad. In fact, the decrease in storage modulus after 200 °C is probably due to the fact that the sample is not completely held anymore. This causes the measured strain to seem higher and thus explains the decrease of storage modulus.

Therefore, the sharp decrease in storage modulus, and increase in damping, is rather a loss of signal because the sample is not maintained anymore, and this loss of signal

occurs in the same temperature range as the DSC second endothermic peak, at which the shrinking occurs.

DSC allowed to detect the denaturation temperature at ~ 75 °C. In this case, a slight drop of storage modulus and a clear peak in the loss modulus are visible around that temperature. It is however difficult to interpret why a molecular denaturation results in such a moduli behavior.

Duraform[®] Dural Graft Implant, another collagen pad, was also analyzed with DMA. The moduli and damping evolution with temperature are shown in Figure 3.26 and compared with the ones of HemopatchTM's collagen. At room temperature, Duraform[®] has a higher storage modulus than HemopatchTM's collagen by one order of magnitude, which might indicate that the former is more rigid, but again, neither value can fully be trusted as they depend on the samples' geometry. As temperature increases, the moduli and $\tan \delta$ show a quite similar behavior for both collagen pads. The storage modulus' drop for Duraform[®] occurs a bit earlier however, which might indicate that it shrinks at a slightly lower temperature than HemopatchTM's collagen. Furthermore, the slope is sharper, which might hint at a faster shrinking, but this cannot be asserted. The shrinking temperature and rate could be investigated with other techniques and confirmed with DSC.

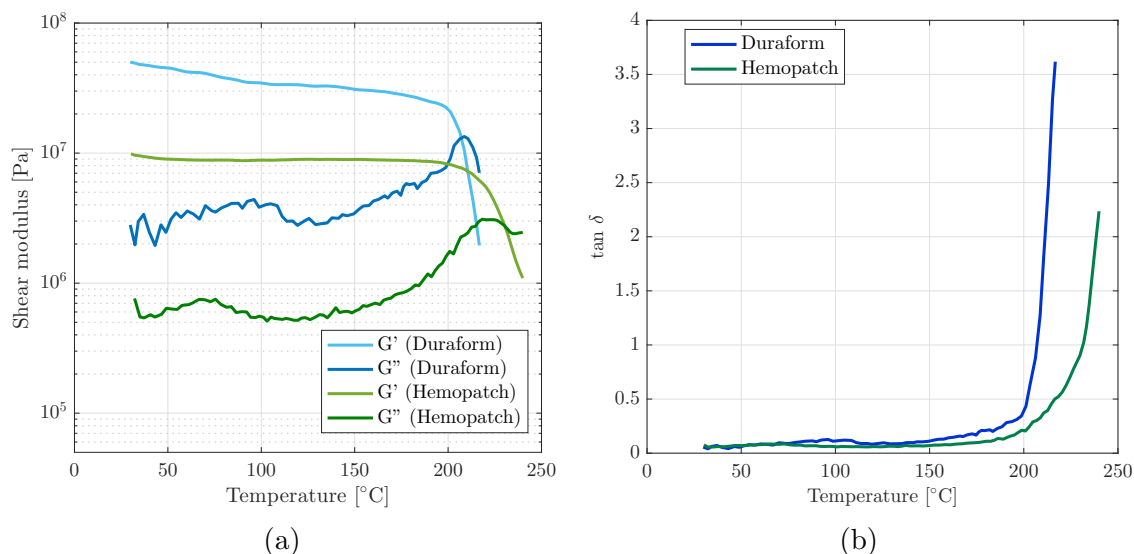


Figure 3.26: Evolution of the storage modulus G' , loss modulus G'' and damping $\tan \delta$ with temperature of Duraform[®] Dural Graft Implant's collagen and of HemopatchTM's collagen

In the end, DMA does not provide reliable information with regards to the mechanical integrity of the samples, as the samples shrink and are therefore not maintained anymore, preventing an accurate monitoring of the moduli. However, the loss of signal due to the shrinking occurs at the same temperature as the DSC second endothermic peak, a little above 200 °C. Thus, DMA in this case provides a rather structural information, matching and confirming the shrinking observations made earlier. It does however not allow to explain the origin of the endothermic peak a little above 200 °C.

3.3.7 Change in pad thickness with temperature

In order to confirm that the decrease of storage moduli measured with DMA, shown in Figures 3.25a and 3.26a, are due to a change in geometry, thermomechanical analysis (TMA) was used in contact mode to record the evolution of the samples' respective thicknesses with temperature, shown in Figure 3.27.

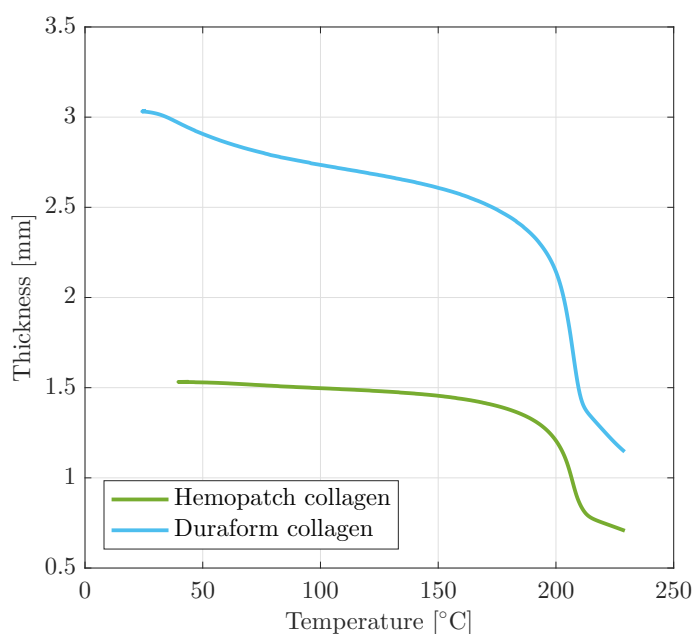


Figure 3.27: Evolution of the relative thickness of two collagen pads with temperature measured with TMA

Both pads' thicknesses decrease at ~ 200 °C, confirming that the decrease in storage modulus, which occurs in the same temperature range, is due to this significant shrinking. HemopatchTM's collagen starts with an initial thickness of 1.5 mm at 30 °C, instead of ~ 2 mm, because the small force applied to monitor the change in thickness compresses the pad a little. Its final thickness at 230 °C is 0.7 mm, which corresponds to what was found as well when the pad was heated in the oven at 225 °C for 4 minutes.

No change in the sample's thickness is observed with TMA at ~ 75 °C, when the first endothermic peak is recorded with DSC and when the loss modulus presents a peak, as recorded with DMA. This again confirms the idea that the pad is only damaged on a molecular level at ~ 75 °C, but that the pad's structural integrity remains intact, as no shrinking occurs.

3.3.8 Mass loss evolution with temperature

As mentioned in Section 3.3.2, the mass of the sample decreases when it is heated. After heating the pad at 225 °C for 4 minutes, the pad loses around 12 % of its mass. Thermogravimetric analysis (TGA) was used to further investigate this mass loss and see how the sample's mass evolves with temperature. Furthermore, in order to confirm the conclusions made in Section 3.3.5 and better understand what happens at ~ 212 °C with DSC, TGA was also used to investigate what the cause of the second endothermic peak might be.

An unmodified sample was thus heated from 30 up to 400 °C at a heating rate of 10 °C/min. The sample's mass evolution is shown in Figure 3.28.

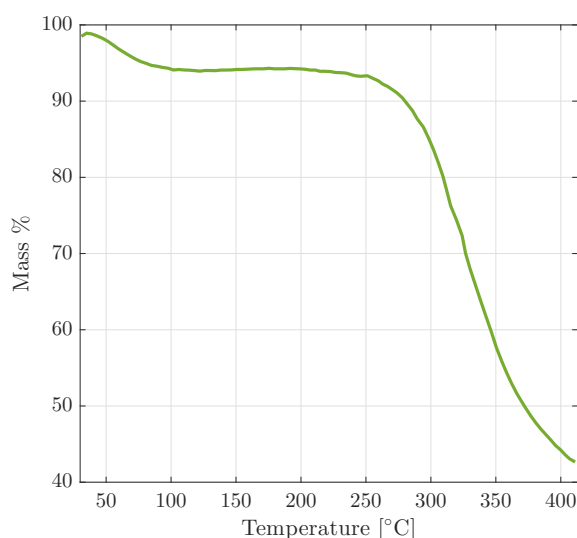


Figure 3.28: Mass evolution of Hemopatch™'s collagen pad with temperature, measured with TGA

At 75 °C, temperature around which the collagen denatures, the pad has lost 5 % of its mass. This confirms that no major structural change has taken place at this point, and that the DSC endothermic peak corresponds to the collagen's denaturation on a molecular level. The 5 % mass loss is likely due to water evaporating from the collagen sample when it is heated. However at 212 °C, temperature at which the second endothermic peak occurs and around which the pad significantly shrinks, the pad has only lost 6 % of its mass, meaning that no significant additional change in mass is noticeable at this temperature either. The 6 % mass loss is again probably due to water evaporation from the collagen. The fact that a structural degradation at 212 °C takes place without a loss in mass might indicate that physical crosslinks in the collagen pad are destroyed at this temperature, explaining the physical damage and shrinking observed, and the endothermic peak at 212 °C. However, this would need to be further investigated in order to truly conclude what causes the shrinking to occur and explain why a DSC endothermic peak is present at this temperature.

At 330 °C, the pad has lost 30 % of its mass, but at that point, the pad is completely degraded and burned.

3.3.9 Thermal evolution of molecular structure by FT-IR spectroscopy

As mentioned, the chemical degradation of the collagen pad is not at the heart of this project but FT-IR spectra of the collagen pad were generated at different temperatures and compared, to serve as an initial exploration of the potential chemical degradation of collagen with temperature.

Figure 3.29 shows the FT-IR spectra of the collagen pad at room temperature and at the three highest measured temperatures: 175 °C, 200 °C and 225 °C. Clearly, at 225 °C, the sample has been heated in such a way that the spectrum is not interpretable anymore meaning that there are no features or bands anymore. Almost no absorption occurs, more than 90 % of the light is transmitted.

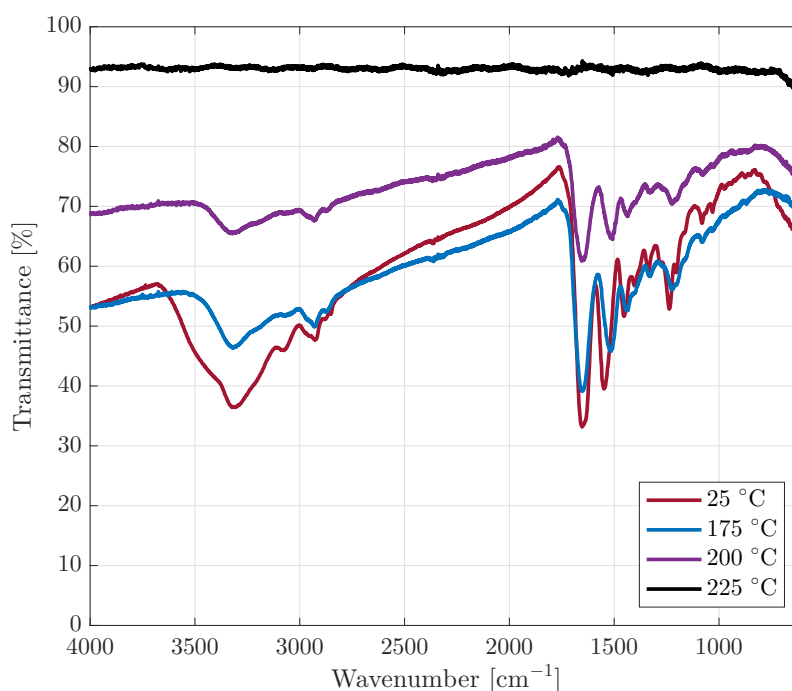


Figure 3.29: FT-IR spectra of collagen pad between 25 °C and 225 °C

At 200 °C, the intensity range between the bands and the baseline is not high enough anymore. Both spectra at 200 °C and at 225 °C correspond to a collagen pad which has gone through a thermal transition and is degraded, as confirmed by the behavior of the spectra. They will therefore not further be taken into account, as no features can be analyzed from those spectra. The spectra will thus be analyzed and compared between 25 °C and 175 °C, as shown in Figures 3.30 and 3.31.

As explained in Section 1.1.1, collagen is a protein, which is a complex molecule made up of a number of different amino acids. This molecule complexity leads to broad, overlapping bands and therefore difficult to differentiate. [65] In the spectrum at 25 °C shown at different magnification levels in Figures 3.29, 3.30 and 3.31, a few typical amino acid bands - and therefore by extension associated to collagen itself - can be recognized at the following wavenumbers:

- 3321 cm^{-1} : absorption due to N-H stretching (usually around 3300 cm^{-1}),
- 1655 cm^{-1} : absorption of amide I, mostly due to CO stretching (usually around 1655 cm^{-1}),
- 1549 cm^{-1} : absorption of amide II due to NH bending and CN stretching (usually around 1565 cm^{-1}),
- set of 3 smaller bands centered at 1237 cm^{-1} : amide III vibration modes (usually around 1245 cm^{-1}). [55] [65]

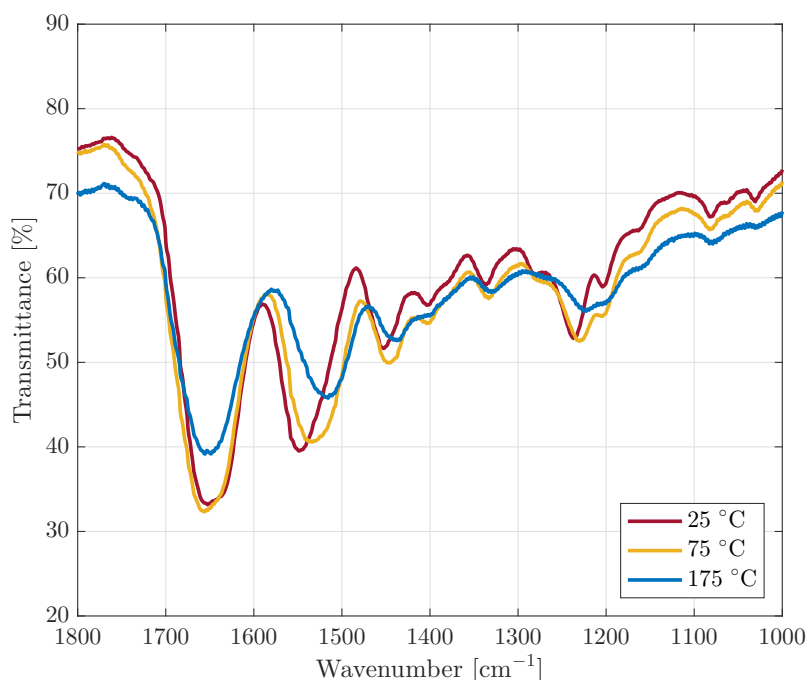


Figure 3.30: Region of FT-IR spectrum of collagen pad at 25, 75 and 175 °C

Comparing the spectrum at 25 °C with the ones at 75 and 175 °C in Figure 3.30, it looks like most bands become weaker in amplitude but also that the entire spectrum shifts towards lower wavenumbers. This is especially clear when looking at one specific band, for instance the one at 1549 cm^{-1} at 25 °C, presumably corresponding to a secondary amide, which moves to 1534 cm^{-1} at 75 °C and to 1516 cm^{-1} at 175 °C. Figure 3.31 shows this band zoomed-in and with all the spectra recorded at the temperatures in between. These confirm that there is a switch occurring towards lower wavenumbers as temperature increases.

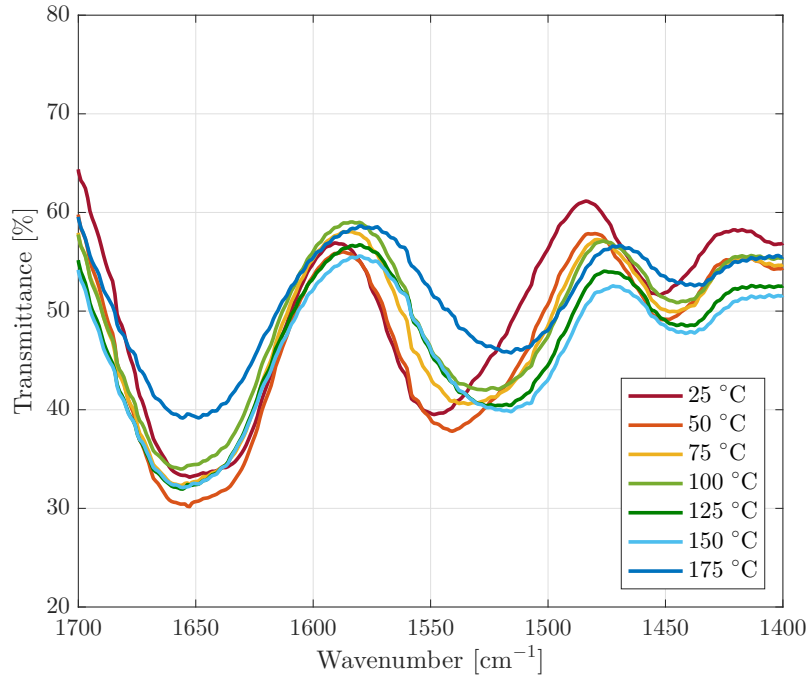


Figure 3.31: Region of FT-IR spectrum of collagen pad between 25 °C and 175 °C, showing a switch of the band at $\sim 1549 \text{ cm}^{-1}$ towards lower wavenumbers as temperature increases

In literature, FT-IR spectroscopy has been used to detect changes in the tertiary structure of collagen, namely its triple helix structure. More specifically, reports on collagen indicate that the infrared absorption ratio $\frac{I_{1235}}{I_{1450}}$ is 1 for pure collagen and that denatured collagen shows a ratio of 0.59, indicating the loss of triple helicity, which has been referred to as collagen *denaturation* in this thesis. [28]

This reasoning was attempted for the spectra of the collagen pad at 25, 75 and 175 °C, where both peaks at 1235 and 1450 cm^{-1} are present, just at slightly different wavenumbers. As DSC heat curves present an endothermic peak at 75 °C, which has been attributed to the collagen molecular *denaturation*, it is interesting to compare this with the intensity ratio $\frac{I_{1235}}{I_{1450}}$ at 75 °C. A comparison with the ratio at 175 °C is made as well, as it is the highest temperature at which a interpretable spectrum was recorded, closest to the temperature at which the pad structurally degrades.

The intensity ratio was calculated in two different ways. On the one hand, the intensity ratio of the bands was calculated as the ratio of the areas above the curve i.e. between the spectrum (in red) and the blue line as shown in Figure 3.32. The integration limits were adjusted for the bands at 75 and 175 °C, since the entire spectrum has shifted towards lower wavenumbers, in order to truly integrate the area corresponding to the band. On the other hand, the second calculation was done by taking the ratio of the peak height of both bands as represented by the green straight line in Figure 3.32. Both ways are commonly used to compare FT-IR peak intensities.

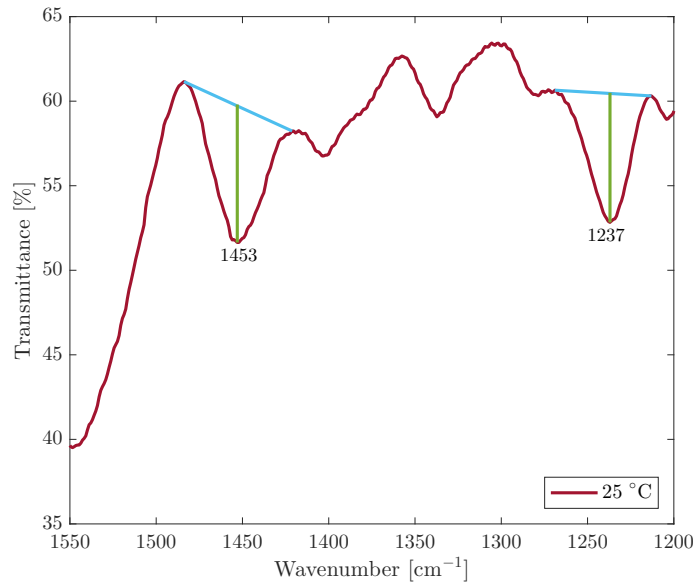


Figure 3.32: FT-IR spectrum with elements for calculation of intensity ratio $\frac{I_{1235}}{I_{1450}}$

The results of this intensity ratio calculation are shown in Table 3.6. Both ways of calculating the ratio - whether using the areas or the band height - lead to an intensity ratio $\frac{I_{1235}}{I_{1450}}$ which in fact decreases as temperature increases, in accordance with literature prediction. At 25 °C, the peak height ratio is 0.94, which is, as predicted in literature close to one, and would correspond to a pure collagen. On the other hand, and again in accordance with literature, this ratio decreases to 0.71 at 75 °C, and to 0.64 at 175 °C, possibly indicating the loss of collagen triple helicity. The ratio obtained when using the area above the bands also decreases as temperature increases: from 0.79 at 25 °C, to 0.61 at 75 °C, and finally 0.51 at 175 °C. This is however not as close to the predicted results in literature.

Table 3.6: FT-IR bands intensity ratio $\frac{I_{1235}}{I_{1450}}$

	25 °C		75 °C		175 °C	
	Area [a.u.]	Peak height [%]	Area [a.u.]	Peak height [%]	Area [a.u.]	Peak height [%]
Band at ~1235 cm ⁻¹	195	7.58	117	4.49	44	1.90
Band at ~1450 cm ⁻¹	248	8.07	193	6.28	86	2.99
Ratio	0.79	0.94	0.61	0.71	0.51	0.64

Ultimately, any interpretation made here above definitely has to be taken with a certain degree of uncertainty. No in-depth FT-IR analysis has been done on the collagen pad and as mentioned, this serves as an initial analysis of chemical degradation. In order to truly make any conclusion on the chemical degradation of the collagen pad, other analysis methods should be used to compare the different results. Furthermore, an intensity ratio can be calculated in many different ways and therefore yield many different results. Finally, the collagen pad is a very complex material, with an intricate supramolecular structure and is therefore difficult to accurately analyze chemically.

CONCLUSION

HemopatchTM Sealing Hemostat is a NHS-PEG-coated collagen pad and hemostatic agent designed to help stop or limit bleeding during surgery. This thesis aimed at characterizing the porous structure of the collagen pad and finding ways to quantify its complex porosity, as no information regarding the pad's fabrication process or its structural properties are available. Additionally, the second leading objective of this thesis was to examine the collagen pad's structural alterations with temperature and understand their origins or causes. This might provide a way to modify the pad's properties post-manufacturing.

The collagen pad comprises a highly porous scaffold, with an estimated relative density between 1 and 3 %. Partly owing to this very low occupied volume, cutting cross-sections of the pad proved to be very challenging and was not managed without damaging the sample. On the other hand, micro-CT allows to obtain a 3D-visualization of the collagen pad, as well as a number of parameters to quantify the pad's porosity and structure, unfortunately only taking into consideration fibers with a diameter above 1 μm . With this limitation acknowledged, it appears that 86 % of the fibers have a diameter between 3 and 9 μm . The 14 % remaining are divided in 8 % between 1 and 3 μm and 6 % between 9 and 33 μm . Additionally, the structure is rather isotropic with a DA of 0.33. The slight anisotropy relies on a preferential direction of the fibers within the xy-plane, thus parallel to the pad's surface. Finally, particle size analysis indicates that most pores of the scaffold have a diameter above 10 μm , a low interconnectivity and that the pad itself generates non-spherical residues when water is filtered through it, part of these definitely larger than 10 μm in one direction.

Concerning the pad's alterations with temperature, it is known that collagen shrinks when it is heated. This interesting property could be exploited for optimizing the pad's hemostatic and sealing properties. Firstly, different degrees of shrinking can be obtained by adjusting both the time and temperature of heating. Their respective effect on the collagen scaffold could further be investigated as to find the structure which provides the most adequate properties for hemostasis.

In addition, as the pad shrinks, it is coherent that its relative density increases. While the mass of the pad seems to slightly decrease due to water evaporating from the collagen, the decrease in volume is much more significant. Thus, the relative density of the pad increases from 1 - 3 % to ~ 46 % after heating the pad at 225 °C for 4 minutes in an oven. This means that the occupied volume is much higher. Furthermore, modifying the pad's pore-size and relative density would provide a way of customizing the pad's stiffness as well.

The pore size was found to decrease but most pores seem to maintain a diameter larger than 5 μm . In addition, the rather isotropic structure of the pad was found to shrink isotropically. Subsequently, the pad's thermal transitions were analyzed through different thermal analytical techniques, allowing to detect two major transitions: a molecular *denaturation* at ~ 75 °C, likely owing to the breaking of the collagen triple helix H-bonds, and a structural *degradation* at ~ 210 °C, corresponding to the significant shrinking of the pad. Both appear to be irreversible.

Collagen thermal transitions, degradation or denaturation still remain a subject of debate with many unresolved questions after ~ 40 years of investigation. As a follow-up to this thesis, it would be interesting to scan the degraded pad with micro-CT to quantify other aspects of the structural alterations caused by heat, just like it was done for the unmodified pad. The chemical degradation could also further be investigated. For instance, knowing that a transition occurs at 75 °C and is probably chemical as no structural variation occurs, it would be interesting to record FT-IR spectra between 25 and 125 °C and heating more slowly (5 °C/min) to focus on this region of interest. Furthermore, as the D-period of the unmodified collagen was very well detected and measured with SEM, it would also be interesting to characterize its evolution at different stages of the pad shrinking, in order to see whether a change in D-period occurs at ~ 75 or ~ 210 °C.

Heating the pad with a pre-heated grid served as a preliminary experience to show that it is possible to partially degrade the pad, and obtain regions with different pore sizes and therefore various stiffnesses. Other experimental designs could be devised in order to modify the pad's structure and properties. In fact, pores of a scaffold used in hemostasis should be large enough to allow nutrients diffusion and cell migration, but smaller pores favor cell attachment and might prevent leaking in the hemostatic process. Thus, a next important step would be to identify the optimal pore sizes for these different factors involved in hemostasis and design a way of partially heating and degrading the pad to obtain the adequate pore sizes accordingly.

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