BRNO UNIVERSITY OF TECHNOLOGY
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FACULTY OF CHEMISTRY
FAKULTA CHEMICKÁ

INSTITUTE OF MATERIALS SCIENCE
ÚSTAV CHEMIE MATERIÁLŮ

THE EFFECT OF BIOLOGICALY ACTIVE SUBSTANCES ON THE STRUCTURE AND PROPERTIES OF COLLAGENOUS SUBSTRATES

VLIV BIOLOGICKY AKTIVNÍCH LÁTEK NA STRUKTURU A VLASTNOSTI KOLAGENOVÝCH SUBSTRÁTŮ

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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Zadání diplomové práce:

1) Literární rešerše na téma porézní kolagenové substráty pro buňky a léčiva aplikované v tkáňovém inženýrství
2) Příprava kolagenových substrátů s aditivy zvyšující jejich biologickou aktivitu
3) Chemicko-fyzikální charakterizace připravených vzorků
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6) Závěr

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ABSTRACT

The thesis deals with the preparation of 3D porous collagen scaffolds by freeze-drying and their modification with bioactive compounds. The natural polysaccharides, chitosan, calcium oxidized cellulose and chitin/chitosan-glucan complex for the modification have been used. The mechanical properties of the scaffolds have been enhanced by crosslinking process with carbodiimides. Growth factors have been delivered in the form of platelet lysate.

The influence of biologically active additives, crosslinking agents, and enrichment with growth factors on the properties of the prepared scaffold and their bioactivity in tissues of living organisms have been investigated. Specifically, this study includes the morphological properties, structure, porosity, swelling stability, chemical composition, temperature of denaturation and biological properties. Scanning electron microscopy, infrared specktroscopy, differential scanning calorimetry and confocal microscopy have been used to the characterization.

Prepared collagen substrates involving bioactive additive and platelet lysate could be used as scaffold for growing cells in systems with low mechanical loading and which has potential application in biomedicine.

ABSTRAKT

Diplomová práce se zabývá přípravou 3D porézních kolagenových skafoldů metodou lyofilizace a jejich modifikací bioaktivními látkami. K modifikaci byly použity přírodní polysacharidy – chitosan, vápenatá oxidovaná celulóza a chitin/chitosan-glukanový komplex. Mechanické vlastnosti skafoldů byly upraveny síťováním pomocí karbodiimidů. Růstové faktory byly dodány formou destičkového lyzátu.

Byl zkoumán vliv biologicky aktivních aditiv, siťovacího činidla a obohacení růstovými faktory na vlastnosti připravených skaffoldů a jejich bioaktivitu v tkáních živých organismů. Konkrétně byly studovány morfologické vlastnosti, struktura, porozita, botnání, stabilita, chemické složení, teplota denaturace a biologické vlastnosti. K charakterizaci byly použity metody rastrovací elektronová mikroskopie, infračervená spektroskopie, diferenční kompenzační kalorimetrie a konfokální mikroskop.

Připravené kolagenové substráty obohaceny bioaktivním aditivem a destičkovým lyzátem mohou být využity v biomedicíně jako skafoldy pro růst buněk v systémech s nízkou mechanickou zátěží.

KEY WORDS

Tissue engineering, scaffold, collagen, freeze-drying.

KLÍČOVÁ SLOVA

Tkáňové inženýrství, skafold, kolagen, lyofilizace.

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DECLARATION

I declare that my diploma thesis was worked out independently and that the used references are quoted correctly and fully. The content of the above mentioned thesis is considered a property of BUT Faculty of Chemistry and can by used for commercial purposes only with the supervisor's and dean's consents.

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author's signature

PROHLÁŠENÍ

Prohlašuji, že jsem diplomovou práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Tato práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího diplomové práce a děkana FCH VUT.

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podpis diplomanta

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CONTENT

1 INTRODUCTION

Collagen as a material for tissue engineering has long been studied for its unique biological and mechanical properties similar to the extracellular matrix. It is obtained from renewable sources which are physiologically intimate or almost identical to collagen in the human body. Creating a 3D porous structure, collagen may be used as a scaffold, which is able to substitute soft tissues. However, collagen has imperfections which can be eliminated by combination with other biomaterials. Many researches are aimed at modifying of collagen scaffolds using biodegradable polymers. Then the resulted scaffold exhibits suitable mechanical properties, stability and a certain biocompatibility required for regeneration of damaged tissue. The bioactivity is here the missing feature, which mediates the matrix interaction with live tissue and becomes indispensable component during regeneration of damaged tissues.

By collagen scaffold modification using biologically active substances, the scaffold is able to provide not only supportive environment for cell growth of new tissue, but also accelerate the growth. Activity means the inclusion of additive substances into the metabolic processes of cells. Substances which were choosen to modify collagen scaffolds are oxidized cellulose, chitosan, and chitin/chitosan-glucan complex. These are polysaccharides that are derived from natural sources, they are biodegradable and exhibit biological activity.

Complete tissue regeneration occurs as supplies of signals which initiate the healing process, the proliferation of cells and mediate communication between the scaffold and undamaged cells. Here it is necessary to use growth factors derived from autologous tissue, otherwise it may occur allergic reactions. An effective method has been proposed to use blood lysate due to high content of growth factors and ease of preparation.

By combining the collagen, bioactive agents and growth factors, a proper scaffold is prepared. It can replace the extracellular matrix and is a suitable environment for adhesion and proliferation of cells. The scaffold performs these functions for a period of recovery, which gradually degrades while forming new tissue structure.

Comparing the results obtained from the prepared scaffolds, the use of scaffolds for tissue engineering and further adjustment will be able to better specify.

2 CURRENT STATE OF THE ART

2.1 Regenerative Medicine

Regenerative medicine is a broad interdisciplinary field defined by the goal of reconstructing, repairing, or replacing missing or damaged tissue (cells, organs) to a state as close as possible to its native architecture and function. Goal should always be to restore the patients to a state of natural form and function through our surgical interventions [1].

In regenerative medicine, cell therapies are the major interest. The main aim of cell therapy is to replace the biological function of demaged tissue or organs. This can be achieved by the transplantation of isolated and characterized cells to a target organ in sufficient number and quality for them to survive long enough to restore function [2].

Development of technology to enable transplanted cells to be engrafted for a long time is indispensable for maximizing the effects of regenerative medicine. As a simple method appears the transplantation of cells as a single cell suspension, but many cells are known to be lost soon after transplantation, which leads to marginal effects. Tissue engineering is a promising strategy to overcome these problems [3].

2.2 Tissue Engineering

Tissue engineering means the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function [4].

Tissue engineering combines the use of i) cells, ii) signals and iii) scaffolds for the purpose of tissue/organ repair/regeneration (Figure 1).

i) The diversity of cell-based applications has dramatically increased with both somatic and stem cell populations being used for tissue engineering. They are being derived from sources throughout the body and our expanding knowledge of cell plasticity and fate has allowed for greater control over their behavior [5].

ii) Signals means biological factors, which are tissue-inducing substances and plays a critical role in organ morphogenesis and regeneration, and refer to a controlling cell behavior and direct tissue and organ repair [6].

iii) Engineered scaffolds primarily function as the extracellular matrix holding tissues together, but they have increasingly been used for cell and/or biomolecule delivery as well.

Creating functional multi-tissue constructs is difficult, because generating manner through cell-cytokine-matrix interactions must be controlled over time and space.

In the following will be discussed the combined use of cells, scaffolds, and inductive therapies for tissue engineering.

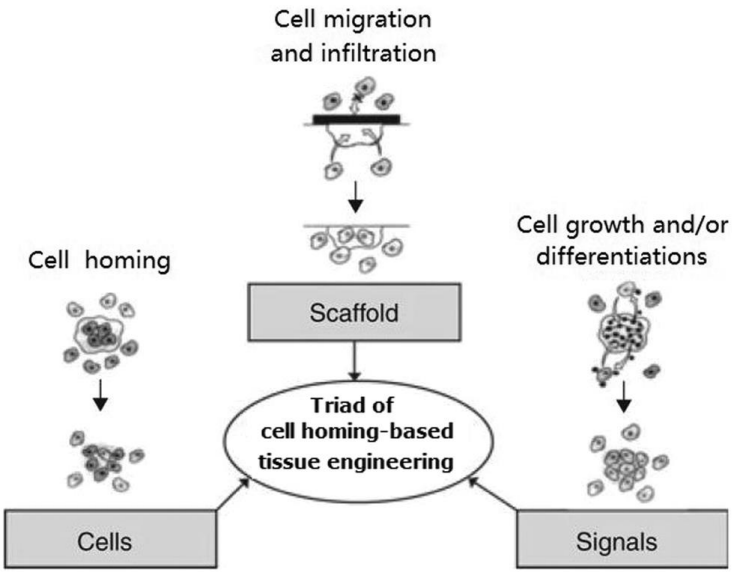


Figure 1: The triad of cell homing-based tissue engineering. To generate functional biohybrid prostheses from these substantial components, specific biologic signals provide desired phenotypes and behaviour of the cell [7].

2.3 Cells

Cells constitute the fundamental unit of tissues and exhibit a broad spectrum of functional diversity. Two main categories of cellular components will be considered for use in tissue engineering: stem and progenitor cells and mature differentiated cells.

Adult stem cell populations are found in almost every tissue and play a pivotal role in tissue homeostasis [8]. Adult stem cells have the capacity to restore and regenerate tissues and are already programmed to sustain a specific tissue type [9]. Differentiated adult cells perform discrete and highly specialized functions that define a particular tissue. They have the advantage of already being defined toward a specific lineage and do not require specific manipulation to perform a tissue-specific task. By contrast, they lack intrinsic regenerative potential and thus are potentially limited in their usefulness for tissue engineering. Anyway, paracrine communication between stem cells and differentiated cells is likely important to maintain the proper function of each subpopulation and these codependent interactions can potentially be augmented to enhance functional tissue engineering [5].

Stem cell is a cell with the ability for self-renewal and diferentiantion potential. Self-renewal means asymmetric cell division which leads to at least one daughter cell which is equal to mother cell [10]. Differentiation refers to the ability of a stem or progenitor cell to form a mature lineage cell.

Progenitor cell is partially differentiated cell which can proliferate and has the capacity to differentiate but with no self-renewal ability.

Potency is defined as the ability to differentiate into mature lineage cells and tissues [11]. There are different stem cell types possess varying degrees of potency [10]:

- Totipotent cells – form whole organism, e.g. zygote, a fertilized egg.
- Pluripotent cells – form all germ layers i.e. ectoderm, mesoderm and endoderm, e. g. embryonic stem cells.

- Multipotent cells – form multiple cell lineages which create an entire tissue, usually specific to one germ layer, e.g. adult stem cells - hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs).
- Oligopotent cells – form two or more cell lineages in a tissue, e. g. neuronal stem cells with ability to form different neurone types.
- Unipotent cells – form one cell lineage, e.g. spermatogonial stem cells, intestinal crypt stem cells, follicular bulge skin stem cells.

Plasticity refers to the controversial possibility for adult stem cells to show higher potency in response to different microenvironments. The niche is cellular microenvironment providing support and stimuli to control stem cell properties [10].

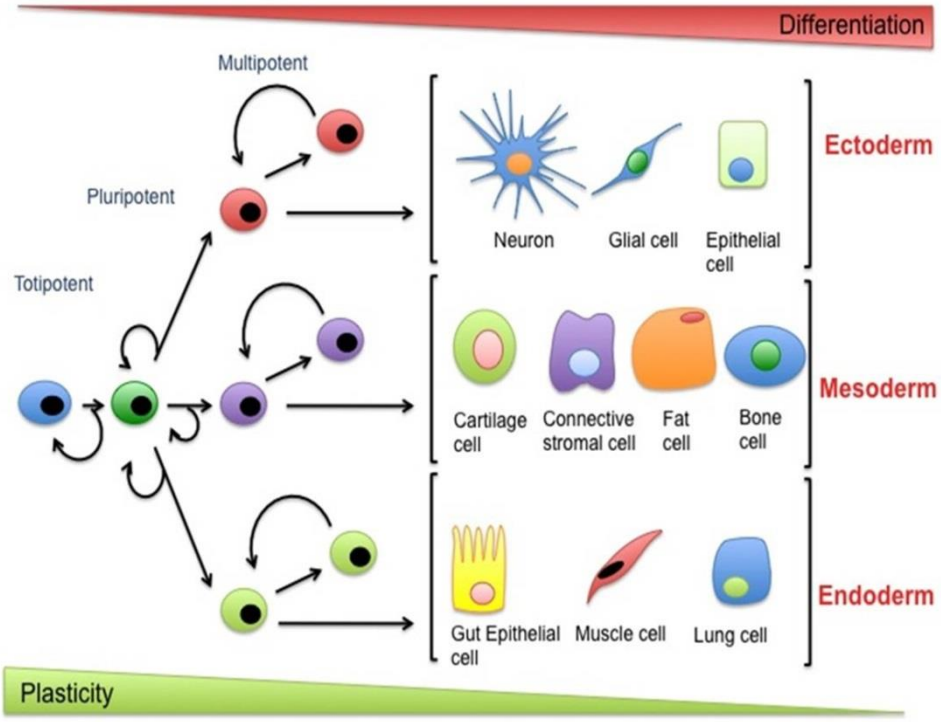


Figure 2: Schematic of different stem cell types divided by the potency and their differentiantion potential [12].

Other cell types used for tissue engineering include embrionic stem cells (ESCs) which can differentiate into tissues from all 3 germ layers but are complicated by the potential for tumorigenesis and ethical concerns [13]. The development of induced pluripotent stem (iPS) cell technology promises to provide tissue engineers with an entirely new set of tools to fabricate organs [14].

In summary, a diverse population of cells critically maintains normal organ homeostasis and these cells and their interactions must be tightly regulated to successfully engineer functional tissue/organ constructs.

2.4 Signals

While a primary requirement of tissue engineering is to select the optimal cell types and scaffold properties to reconstruct a specific tissue in its particular configuration and function, a pivotal variable for succesful regeneration is the cellular microenvironment in which cells and tissue grow [15].

Cell fate is influenced by a number of factors and interactions that require robust control for safe and effective regeneration of functional tissue. Coordinated interactions with cellular microenvironment that cells sense and that performs complex and dynamic regulation of cellular processes. The basis for tissue regeneration, therfore, is the utilization of engineering techniques that mimic the critical aspects of natural healing processes, „the wound healing cascade“ by providing suitable biochemical and physico-chemical factors [16]. The self-healing capacity of patients can be augmented by artificially accelerating the proliferation and differentiation of the recruited or implanted cells via the integration of growth factors (GFs) and cytokines [16], [17]. It is indispensable to provide cells with a local biochemical and mechanical niche mimicking the natural environment in which they can proliferate and differentiate efficiently by creating an artificial extracellular matrix (ECM) and/or by delivering GFs [16], [17], [18], [19], [20], [21], [22]. One fundamental approach to tissue engineering has been to create such an environment to mimic the natural „wound healing cascade“ for induction of regeneration and to accelerate/exploit the inherent capability for tissue regrowth that is generally termed regenerative medicine [16]. Within the complex cascade of biological events, GFs are known to play a central role in information transfer between a wide range of cells and their ECM, and stimulating endogenous repair mechanisms by providing the right signale to cells a thereby leading to an accelerated functional restoration of damaged or defective tissues [23], [24], [25], [26].

2.4.1 Platelet-Rich Plasma

Platelet-rich plasma (PRP) is a platelet concentrate in a small volume of plasma that is typically developed from autologous blood [27]. Platelets are cytoplasmic fragments of megakaryocytes and play a central role in the complex process of hemostasis. Clot formation and platelet activation are considered the first steps of the healing proces [28]. Within 10 minutes of blood coagulation, platelets release a burst of proteins from their α, δ, and λ granules. These organelles contribute the numerous biologically active molecules that provide platelets with their healing properties. The contents of the -granule include many growth and differentiation factors that are released upon platelet activation during injury to the vessel wall [29]. The combined effects of these growth factors create an elaborate autocrine and paracrine process, and therefore may result in tissue-specific responses.

Within an hour of activation, 95% of the granule’s previously manufactured growth factors will be secreted. The platelets then sustain production of additional growth factors over the next several days [30].

For the platelets to release this cascade of molecules that contribute to tissue healing, they must first be activated. Some studies activate PRP with thrombin and or calcium [31]. Once activated, the resulting PRP mixture must be injected immediately, as the subsequent secretion of the granule contents occurs rapidly. The addition of thrombin and calcium to the platelet mixture produces a gel that cannot be injected even using a largegauge needle, thus limiting its use in certain surgical procedures.

The application of soluble type-I collagen has been evaluated as an alternative to thrombin and calcium in platelet activation. Activation via type-I collagen produces a less rapid release of the granule contents, which enable a delayed administration of PRP. When compared with thrombin activation, the collagen-activated platelets produced equal concentrations of particular growth factors with less clot retraction. The collagen activation technique also

permits in vivo activation that can be administered through a small-gauge needle. These results suggest that type-I collagen may be an effective method for platelet activation [32].

2.4.1.1 Growth Factors in PRP

Benefits of this therapy with PRP at the molecular level include the intrinsic properties of its growth factors. It is important to note that growth factors are not the only elements present in significant concentrations in PRP, as proteins of the cytokine and chemokine families are known to be present in varying concentrations as well. The elements identified in PRP include TGFβ, vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor, epidermal growth factor, insulin-like growth factor, platelet factor 4, IL-1, platelet-derived angiogenesis factor, epithelial cell growth factor, osteocalcin, osteonectin, thrombospondin-1, fibrinogen, fibronectin, and vitronectin. The concentrations of these various growth factors increase linearly as platelet concentration increases [29], [33]. The network of activated growth factors induces intracellular signaling pathways that lead to the production of proteins essential to the regenerative processes, such as cell proliferation, matrix formation, osteoid production, and collagen synthesis [34].

Cell types that are involved in the healing process, such as osteoblasts, fibroblasts, epithelial cells, endothelial cells, and adult mesenchymal stem cells reveal the presence of membrane receptors that are specific for certain growth factors. Furthermore, when cytokines are released they bind to the transmembrane receptors on the surface of local or circulating cells [28], [35]. At the time of injury, the platelets arrive via the capillaries and are activated, releasing their granule contents into the wound site. This influx of growth factors and proteins play an active role in synthesizing the necessary components for the regenerative process and may also play a paracrine role by recruiting other cells to the wound site.

A summary of these growth factors, their role in the healing proces are presented in Table 1.

Table 1: Effect of the growth factors produced by platelets [36].

Growth factor	Effect
PDGF (platelet-derived growth factor)	Macrophage activation and angiogenesis Fibroblast chemotaxis and proliferative activity Enhances collagen synthesis Enhances the proliferation of bone cells
TGF-β (transforming growth facor-β)	Enhances the proliferative activity of fibroblasts Stimulates biosynthesis of type I collagen and fibronectin Induces deposition of bone matrix Inhibits osteoclast formation and bone resorption
IGF-1 (insulin-like growth factor-1)	Chemotactic for fibroblasts and stimulates protein synthesis Enhances bone formation by proliferation and differentiation of osteoblasts
PDEGF (platelet-derived endothelial growth factor)	Promotes wound healing by stimulating the proliferation of keratinocytes and dermal fibroblasts
PDAF (platelet-derived angiogenesis factor)	Induces vascularization by stimulating vascular endothelial cells
PF-4 (platelet factor-4)	Stimulates the initial influx of neutrophils into wounds A chemoattractant for fibroblasts A potent antiheparin agent
EGF (endothelial growth factor)	Cellular proliferation Differentiation of epithelial cells
VEGF (vascular endothelial growth factor)	Angiogenesis Migration and mitosis of endothelial cells Creation of blood vessel lumen Creates fenestrations Chemotactic for macrophages and granulocytes Vasodilation (indirectly by release of nitrous oxide)

2.4.1.2 Preparation of Platelet-Rich Plasma

The whole blood which is a mixture of cells, colloids and crystalloids can be separated into different blood components namely packed red blood cell (PRBC) concentrate, platelet concentrate, fresh frozen plasma and cryoprecipitate [27], [37].

In increasing order, the specific gravity of blood components is plasma, platelets, leucocytes (Buffy Coat [BC]) and packed red blood cells (PRBCs) [38].

The components are prepared by centrifugation of one unit of whole blood. Single component required can also be collected by apheresis procedure in blood donors.

Different platelet concentrates can be classificated into four categories, depending on their leucocyte and fibrin content:

- pure platelet-rich plasma (P-PRP),
- leucocyte- and platelet-rich plasma (L-PRP),
- pure plaletet-rich fibrin (P-PRF),
- leucocyte and platelet-rich fibrin (L-PRF).

All available PRP techniques have some points in common:

Blood is collected with anticoagulant just before or during surgery and is immediately processed by centrifugation. The time for platelet concentrate preparation is variable but is always completed within an hour. A first centrifugation step is designed to separate the blood into three layers, red blood cells (RBCs) are found at the bottom, acellular plasma (PPP, platelet-poor plasma) is in the supernatant and a 'buffy coat' layer appears in between, in which platelets are concentrated (Figure 3). The next steps vary among the numerous

protocols but are an attempt to discard both the RBC layer and the PPP to collect only the 'buffy coat' layer. Finally, the obtained platelet concentrate is applied to the surgical site with a syringe, together with thrombin and/or calcium chloride (or similar factors) to trigger platelet activation and fibrin polymerization.

In Choukroun's PRF (platelet-rich fibrin) preparation is blood collected without any anticoagulant and immediately centrifuged. A natural coagulation process then occurs and allows for the easy collection of a leucocyte- and platelet-rich fibrin (L-PRF) clot, without the need for any biochemical modification of the blood, that is, no anticoagulants, thrombin or calcium chloride are required [39].

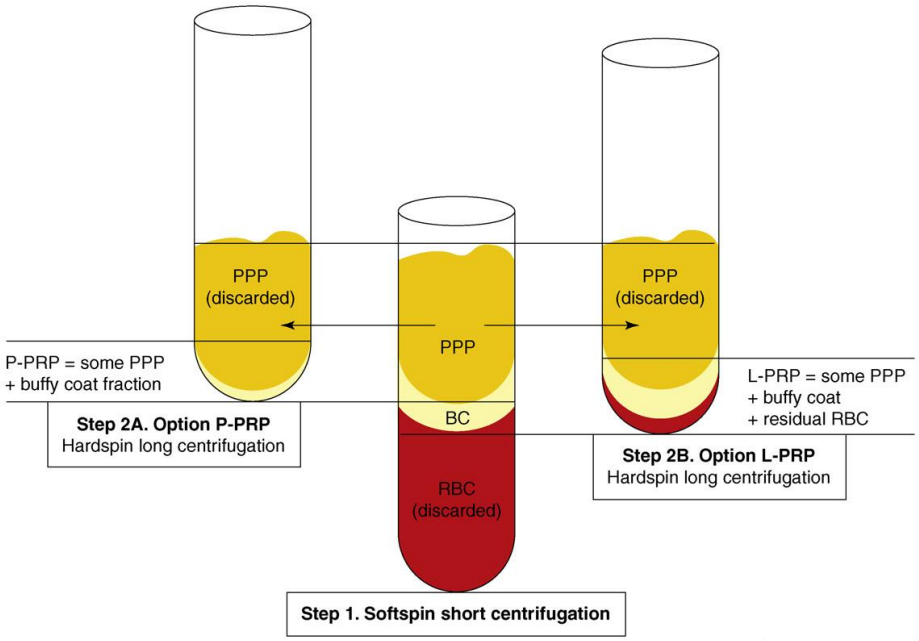


Figure 3: Classical manual platelet-rich plasma (PRP) protocol using a two-step centrifugation procedure [40].

2.4.1.3 Platelet Lysate

Platelets constitute a potential source of multiple GFs and proteins involved in tissue regeneration. Some of GFs are available in purified form, but it has been pointed out that tissue repair cannot be effectively mediated by a single agent, as multiple signals are required to complete the regeneration process. Moreover it is also recognized that the efficacy of the GFs critically depends on the way they are made available to the injured tissue [41].

Platelet-rich preparations are hemoderivatives, from which platelets can release their complete pool of biologically active substances.Among these hemoderivatives is platelet-rich plasma (PRP), which can be obtained from the patient (autologous) or from donors (allogenic) [42], [43], [44], [45].

The platelet concentrate can therefore be activated by adding thrombin or calcium to form athree-dimensional and biocompatible fibrin scaffold (fibringlue). The term 'releasate' is usually used to describe the preparations, which consist of bioactive molecules in a solution obtained by activation of platelets with calcium or thrombin, while the term 'lysate' indicates the solution of bioactivemolecules obtained by platelet destruction by freeze-thawing,usually starting from a PRP sample in the presence of ananticoagulant agent [46].

Platelet lysate promotes keratinocyte epithelialization and regulates fibroblast matrix deposition, providing a molecular basis for the capability of such hemoderivative to heal severe and problematic wounds [47].

Platelet lysate loaded in gel and sponge-like dressings intended for the treatment of mucosal, corneal and skin lesions. Such formulations were capable to enhance in vitro proliferation of fibroblasts and corneal (RCE) cells [48], [49], [50], [51], [52].

2.5 Scaffold

Tissue engineering technologies are based on the biological triad and involve the successful interaction between three components: (1) the scaffold that holds the cells together to create the tissue's physical form; (2) the cells that create the tissue; and (3) the biological signalling molecules, such as growth factors, that direct the cells to express the desired tissue phenotype [53].

Scaffold is the central component that is used in tissue engineering to deliver cells, drugs, and genes into the body. They are most commonly recognized as defined structure biomaterials potentially able to perform useful functions:

(i) promoting cell attachment, survival, proliferation and differentiation while possessing minimum toxicity in the original and biodegraded forms;
(ii) allowing the transport or delivery of gases, nutrients and growth factors;
(iii) offering sufficient structural support while being degradable at appropriate rates for tissue regeneration [54].

The scaffolds provide a three-dimensional structure for cell adhesion, proliferation, differentiation, and secretion of extracellular matrices to guide new tissue formation and regeneration [55]. These scaffolds essentially act as a template for tissue formation and are typically seeded with cells and occasionally growth factors, or subjected to biophysical stimuli in the form of a bioreactor; a device or system which applies different types of mechanical or chemical stimuli to cells [56].

These cell-seeded scaffolds are either cultured in *vitro* to synthesize tissues which can then be implanted into an injured site, or are implanted directly into the injured site, using the body's own systems, where regeneration of tissues or organs is induced in *vivo* [1].

When cells are implanted or seeded into an artificial structure capable of supporting three-dimensional (3D) tissue formation, these structures are called "cell delivery scaffolds," and when drugs are loaded into a 3D artificial porous structure capable of high drug loading efficiency and sustained release of a drug for longer duration, they are called "drug delivery scaffolds" [57].

Different forms of polymeric scaffolds for cell/drug delivery are available (Figure 4):

(i) 3D porous matrix – a highly porous and well interconnected open pore structure that allows high cell seeding density and tissue in-growth; (ii)
(ii) nanofibrous matrix – prepared by electrospinning or self-assembly would provide a better resemblance of the physiological environment [56], [58];
(iii) thermosensitive sol-gel transition hydrogel - physically or chemically cross-linked, water-soluble polymers, which swell to form a gel like substance on exposure to water [59];
(iv) porous microsphere and microparticles - delivering biomolecules such as growth factors, genes, and cells [60].

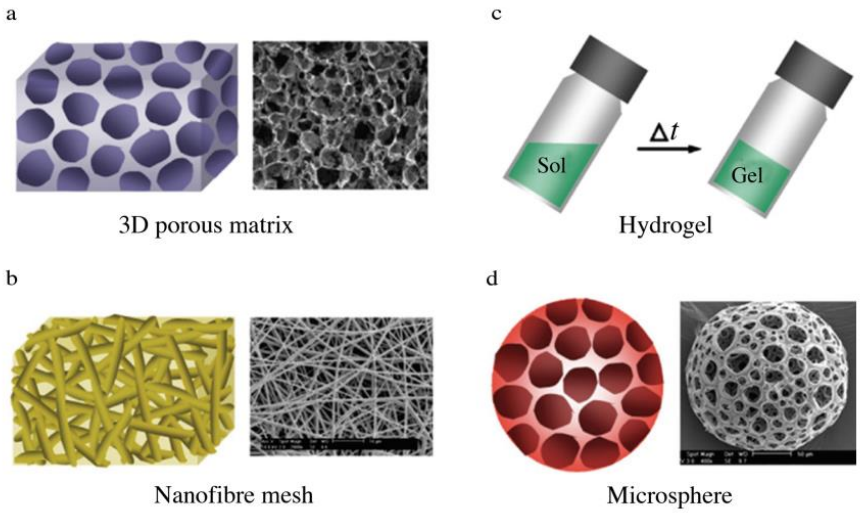


Figure 4: Different forms of polymeric scaffolds for tissue engineering [61].

These are already widely utilized as sustained protein-release formulations and have been applied in tissue engineering for the potential use as a cell delivery carrier or supportive matrix [62], [63].

Of the polymeric scaffolds a typical 3D porous matrix and nanofibrous matrix are the implantable forms and a thermosensitive sol-gel transition hydrogel and porous microsphere are the injectable forms [64].

2.5.1 Properties of Scaffold Matrices

An ideal tissue engineering scaffold (cell delivery) should fulfill the following requirements [65]:

- mechanical properties that are sufficient to shield cells from tensile forces without inhibiting biomechanical cues,
- desired volume, shape, and mechanical strength [58],
- acceptable biokompatibility
- a highly porous and well-interconnected open pore structure to allow high cell seeding density and tissue in-growth
- bioadsorption at predetermined time period
- biocompatible chemical compositions and their degradation products,
- causingminimal immune or inflammatory responses [66],
- physical structure to support cell adhesion and proliferation,
- facilitating cell–cell contact and cell migration [64].

In the section below we will learn more about desired properties.

Mechanical properties of the scaffold should match those of the tissue at the implantation site, or the mechanical properties at least should be sufficient to shield cells from damaging compressive or tensile forces without inhibiting appropriate biomechanical cues [64], [67] and to survive under physiological conditions [53].

Mimicking the native extracellular matrix (ECM), an endogenous substance that surrounds cells, allows them to bind into tissues and provide signals that aid cellular development and morphogenesis [64].

The scaffold should possess relatively easy processability and malleability into the desired shape, according to the need. They should be capable of being produced into a sterile product.

The scaffold should possess acceptable biocompatibility and toxicity profiles. Cells must adhere, function normally, and migrate onto the surface and eventually through the scaffold and begin to proliferate before laying down new matrix. After implantation, the scaffold must elicit a negligible immune reaction in order to prevent it causing such a severe inflammatory response that it might reduce healing or cause rejection by the body [1].

It should have a reproducible microscopic and macroscopic structure with a high surface: volume ratio suitable for cell/drug attachment [68]. Scaffolds should have an interconnected pore structure and high porosity to ensure cellular penetration and adequate diffusion of nutrients to cells within the construct and to the extra-cellular matrix formed by these cells. A porous interconnected structure is also required to allow diffusion of waste products out of the scaffold [1]. Cells primarily interact with scaffolds via chemical groups (ligands) on the material surface. Scaffolds synthesized from natural extracellular materials (e.g. collagen) naturally possess these ligands in the form of Arg-Gly-Asp (RGD) binding sequences, whereas scaffolds made from synthetic materials may require deliberate incorporation of these ligands through another mechanism, for example, protein adsorption. The ligand density is influenced by the specific surface area. This depends on the mean pore size in the scaffold. The pores thus need to be large enough to allow cells to migrate into the structure, but small enough to establish a sufficiently high specific surface, leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold [64].

The scaffold should have a maximum loading capacity so the drug is released continuously for longer duration after insertion into the body. The drug release from the scaffold needs to be controlled to allow the appropriate dose of drug to reach the cells over a given period of time.

Binding affinity must be sufficiently low to allow release of the drug.

They should posses dimensional stability, chemical stability, and biological activity over a prolonged period of time [57].

The scaffold material should be biodegradable, its degradation products should not be toxic and should be eliminated easily from the implantation site by the body [53], eliminating the need for further surgery to remove it [69]. The scaffold's degradation rate should be adjusted to match the rate of tissue regeneration so that it has disappeared completely once the tissue is repaired [68].

The matrix material of the scaffold should be biodegrade at a controllable rate that approximates the rate of natural tissue regeneration and should provoke a minimal immune and/or inflammatory response in vivo [53]. Tissue engineering scaffolds are meant to be colonized by cells and should transmit the chemical and physical cues necessary to ensure adequate tissue growth. Synthetic polymer scaffolds may be used to deliver proteins and growth factors with or without cells locally to enhance tissue repair and regeneration [57].

2.6 Biomaterials for Scaffolds

There are three individual groups of biomaterials: ceramics, synthetic polymers and natural polymers, in the fabrication of scaffolds for tissue engineering.

2.6.1 Natural Polymers

Natural polymers often possess highly organized structures and may contain an extracellular substance, called ligand, which can be bound to cell receptors and thay exhibit biological activity and typically promote excellent cell adhesion and growth. they are also biodegradable and so allow host cells, over time, to produce their own extracellular matrix and replace the degraded scaffold [71]. However, as natural polymers can guide cells to grow at various stages of development, they may stimulate an immune response at the same time. This leads the concerns over antigenic and deliver of diseases for allograft [72].

Natural polymers include alginate, proteins, collagens, gelatin, fibrins, albumin, elsinan, pectin (pectinic acid), galactan, curdlan, gellan, levan, emulsan, dextran, pullulan, gluten, elastin, fibroin, hyarulonic acid, cellulose, starch, chitosan (chitin), scleroglucan, heparin, silk, chondroitin 6-sulfate, and polyhydroxyalkanoates [73].

Calcium Oxidized Cellulose

Cellulose is a polysaccharide synthesized by grasses, woody plants, many forms of algae, fungi and some species of bacteria, namely Acetobacter xylinum. Bacterial cellulose is identical to plant cellulose in chemical structure, but it can be produced without contaminant molecules, such as lignin and hemicelluloses, and does not require intensive purification processes. In addition, it is remarkable for its mechanical strength and biocompatibility, so it has often been applied in tissue engineering [74].

Cellulose in the human organism behaves as a non-degradable or very slowly degradable material. An efficient method for inducing degradability of cellulose is its oxidation.

Oxidized cellulose can be generated by various techniques and by various oxidizing agents, such as $NaClO_2$, CCl_4, nitrogene oxides or free nitroxyl radicals (TEMPO oxidation) [75], [76], [77].

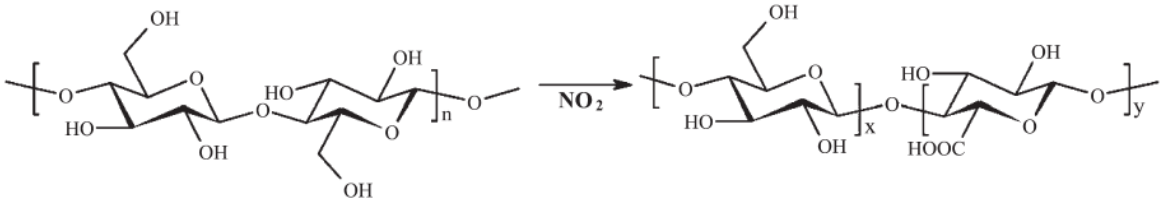


Figure 5: Oxidation proces of cellulose by NO_2 gas [78].

Oxidized cellulose is degradable by hydrolysis, by mediated hydrolytic enzymes present in the serumsupplement of cell culture media in vitro, and in vivo, in macrophages [79].

Cellulose oxidation induces conversion of the glucose residues to glucuronic acid residues containing –COOH groups. The –COOH groups, which are polar and negatively charged, can be used for functionalizing the oxidized cellulose with various biomolecules [80].

Oxidized cellulose has been widely used as a wound healing material with excellent properties, such as high absorbability, antibacterial and antiviral properties, and non-toxic and antiadhesive effects [81]. Due to its ability to initiate or accelerate blood coagulation at the site where it is applied, oxidized cellulose has been used as a hemostatic material [82].

Chitosan

Chitosan is another biomaterial used in a variety of biomedical fields such as drug delivery carriers, surgical thread, and wound healing materials [83]. It is derived from chitin, is

an abundant polysaccharide that may be used to replace glycosaminoglycan [84]. Due to its many advantages for wound healing such as hemostasis, accelerating the tissue regeneration and the fibroblast synthesis of collagen, many applications of chitosan in skin tissue engineering have been reported [85]. In addition, chitosan can function as a bridge to increase the cross-linking efficiency of GA in the collagen-based scaffolds owing to the large number of amino groups in its molecular chain (Figure 6). Hence, one can expect that less GA could be used in the presence of chitosan and the potential cytotoxicity of GA might be decreased [86].

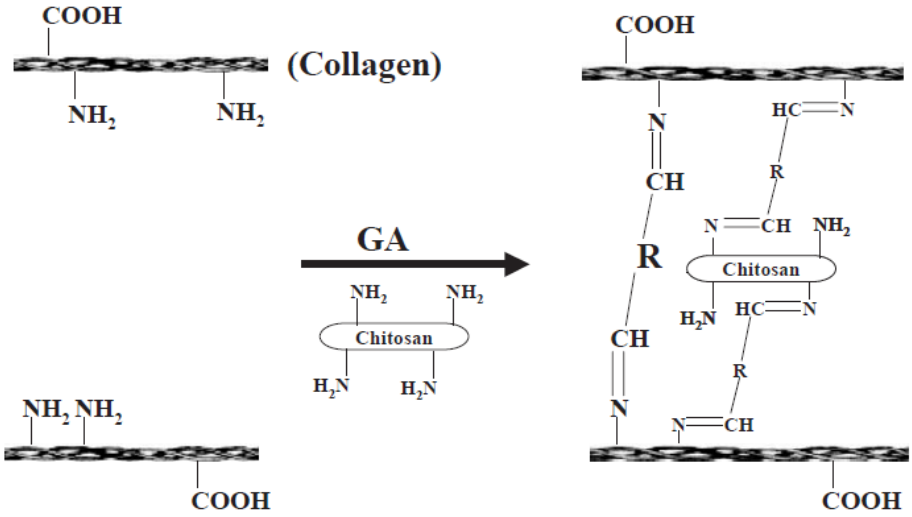


Figure 6: Schematic presentation of collagen cross-linked with glutaraldehyde in the presence of chitosan [86].

Chitin/Chitosan-Glucan Complex

Fungal mycelial wastes from biotechnological plants can become free and rich alternative sources of chitin/chitosan materials, beside the traditional industrial source – shellfish waste materials. Moreover, the fungal chitosans can have unique properties compared with those derived from Crustacea. It have been tested possibilities of production and some applications of chitin-glucan complex and chitosan from industrial waste mycelia of Aspergillus niger from a citric acid production plant and Penicillium oxalicum from production of a red anthraquinone dye in Czech Republic. The alkali-insoluble cell-wall residue of the mycelia consists mainly of chitosan, chitin and ß-glucans, with a significant preponderance of (1→3)-ß-D-glucan. Chitin is thought to be present as microfibrils physically embedded in the ß-glucan matrix. The formation of the chitin/chitosan–glucan complex chains results in a rigid cross-linked network in the cell wall and causes a considerable problem for the extraction of intact chitosan and glucan [87].

2.6.2 Synthetic Polymers

Synthetic polymers are man-made polymers, which have the advantages over the use of natural origin polymers as they are more flexible, more predictable and processable into different size and shapes and hove no immunogenicity [53]. The physical and chemical properties of a polymer can be easily modified and the mechanical and degradation characteristics can be altered by their chemical composition of the macromolecule. The functional groups and side chains of these polymers can be incorporated, i.e. the synthetic polymers can be selfcross-linked or cross-linked with peptides or other bioactive molecules.

Additionally, synthetic polymers are generally degraded by simple hydrolysis that is desirable as the degradation rate does not have variations from host to host, unless there are inflammations and implant degradation etc. to affect the local pH variations [72].

Synthetic polymers are largely divided into two categories: biodegradable and nonbiodegradeable.

Biodegradable polymers are polyglycolide (PGA), polylactide (PLA) and its copolymer poly(lactide-*co*-glycolide), polycaprolactone (PCL), polyethylene glycol (PEG), polyphosphazene, polyanhydride, poly(propylene fumarate), polycyanoacrylate, polycaprolactone, polydioxanone, and polyurethanes.

Nonbiodegradeable polymers include polyvinyl alcohol, polyhydroxyethymethacrylate, and poly(*N*-isopropylacrylamide).

2.6.3 Bioceramics

Melting inorganic raw materials to create an amorphous or crystalline solid body is known as *bioceramics*, and these porous final products are used mainly for scaffolds.

Ceramic scaffolds are typically characterized by high mechanical stiffness (Young's modulus), very low elasticity, and a hard brittle surface. Ceramics are known to enhance osteoblast differentiation and proliferation [88], [89].

Bioceramics classified as (i) nonresorbable (relatively inert), for example alumina, zirconia, and silicon nitride; and (ii) bioactive or surface active (semi-inert), for example glass ceramics such as dense hydroxyapatites [$9CaO{\cdot}Ca(OH)_2{\cdot}3P_2O_5$], and biodegradable or resorbable (noninert) such as calcium phosphates, aluminium calcium phosphates, coralline, tricalcium phosphates ($3CaO{\cdot}P_2O_5$), zinc calcium phosphorus oxides, zinc sulphate calcium phosphates, ferric calcium phosphorus oxides, and calcium aluminates [53].

Collagen-based scaffolds can be also strengthened by introducing a ceramic phase [90]. These scaffolds not only possess significantly increased mechanical properties while retaining the highly porous and interconnected pore structure [91], but also show improved permeability which benefits cell infiltration and subsequent vascularization.

2.6.4 Biocomposites

Because each of the individual biomaterial groups has specific advantages and disadvantages, so the use of composite scaffolds comprised of different phases is becoming increasingly common. Composite scaffolds consists of at least one phase which is not found naturally in the body and they all have associated problems with biocompatibility, biodegradability or both. A more typical approach is the use of collagen-based scaffolds, either alone or with an additional phase incorporated to enhance biological and/or mechanical properties [71].

Combinations of (1) synthetic–synthetic, (2) synthetic–natural and (3) natural–natural polymers have ability to tailor mechanical, degradation, and biological properties but compromise the "best" qualities of individual polymers with properties of the overall scaffold [53].

2.7 Collagen

Collagen had been defined as a family of proteins located in the extracellular matrix of connective tissue that provides a structural basis for the mechanical and biochemical properties of tissues and organs [92].

Several distinct types of collagen had been identified, which collectively, represent about one third of the total protein of vertebrate animals. As a function of structure and supramolecular organization, they were grouped as fibril-forming (types I, II, III, V, XI), fibril-associated (types IX, XII, XIV), membrane (types IV, VII, VIII, X) or other specific function. The fibrilforming collagens are the major structural element of connective tissue, providing the scaffold that gives stability and integrity to tissues and organs [67].

2.7.1 Structure of Collagen

The collagen molecule consists of three polypeptide subunits known as α-chains twined around one another as in a three-stranded rope. Each chain has an individual twist in the opposite direction. The principal feature that affects a helix formation is a high content of glycine and amino acid residues. The strands are held together primarily by hydrogen bonds between adjacent –CO and –NH groups and also by covalent bonds [93].

Currently, 29 distinct collagen types have been characterized and all display a typical triple helix structure [94] which vary in the length of the helix and the nature and size of the nonhelical portions. Type I collagen is predominant in higher order animals especially in the skin, tendon, and bone where extreme forces are transmitted, and also has the advantage of possessing lower antigenic and hypoallergenic properties, therefore it is used as standard in the field of tissue-engineering.

It is a compound of three chains, two of which are identical, termed α1(I), and one α2(I) chain with different amino acid composition or it can rarely represent a trimer built of three α1(I) chains. The basic collagen molecule contains three polypeptide α-chains, each consisting of more than one thousand amino acids [95]. Glycine has the smallest side group and its presence is essential at every third amino acid position in order to allow for a tight packaging of the three α-chains in the tropocollagen molecule and the X and Y positions are mostly filled by proline and 4-hydroxyproline (Figure 7a) [94], [96].

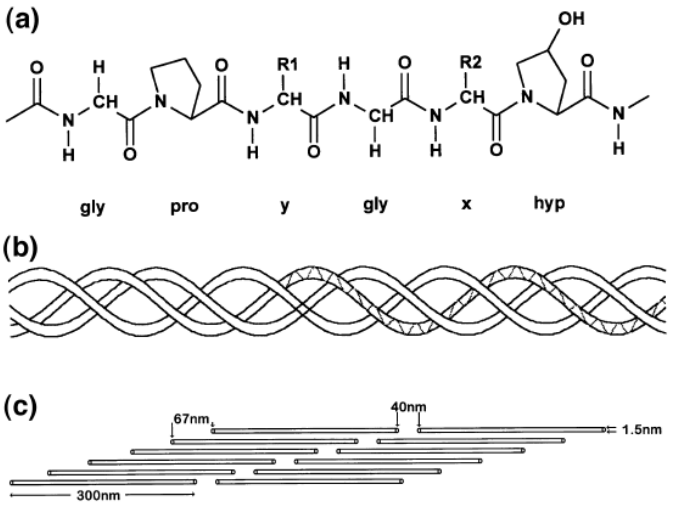


Figure 7: Chemical structure of collagen type I. (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix and (c) staggered quarternary structure [96].

Collagen also contains the unusual amino acid hydroxylysine. Formation of hydroxylysyl residues allows the attachment of sugar components, an unalterable requirement

for the collagen molecule to form a triple-helical structure. Both imino acids stabilize the triple helix. Due to their alicyclic nature, they stiffen the α-chain and form hydrogen-bonds limiting rotation [97].

The unique physiological and biomaterial characteristics of collagen compared with most synthetic polymers derive from the structural complexity of the collagen molecule. There are observed the various levels of order in collagen. [98] The tertiary structure refers to the fundamental unit originally known as tropocollagen: three polypeptide chains intertwined to form a right-handed triple-helix with a pitch of approximately 8.6 nm.

The rod-shaped triplehelix has an average molecular weight of approximately 300 kDa, a length of 300 nm with a diameter of 1.5 nm (Figure 7) [99].

There are regions of 9–26 amino acids at the amino and carboxyl terminal chain ends of the molecule that are not incorporated into the helical structure. These non-helical regions are denoted as telopeptides. On the fourth level of order, the triple-helical molecules associate of individual triple helices longitudinally and bilaterally into fibrils with distinct periodicity.

The collagen molecules aggregate through fibrillogenesis into microfibrils consisting of four to eight collagen molecules and further into fibrils. Those fibrils reach from 10 to 500 nm in diameter depending on tissue type and stage of development [99].

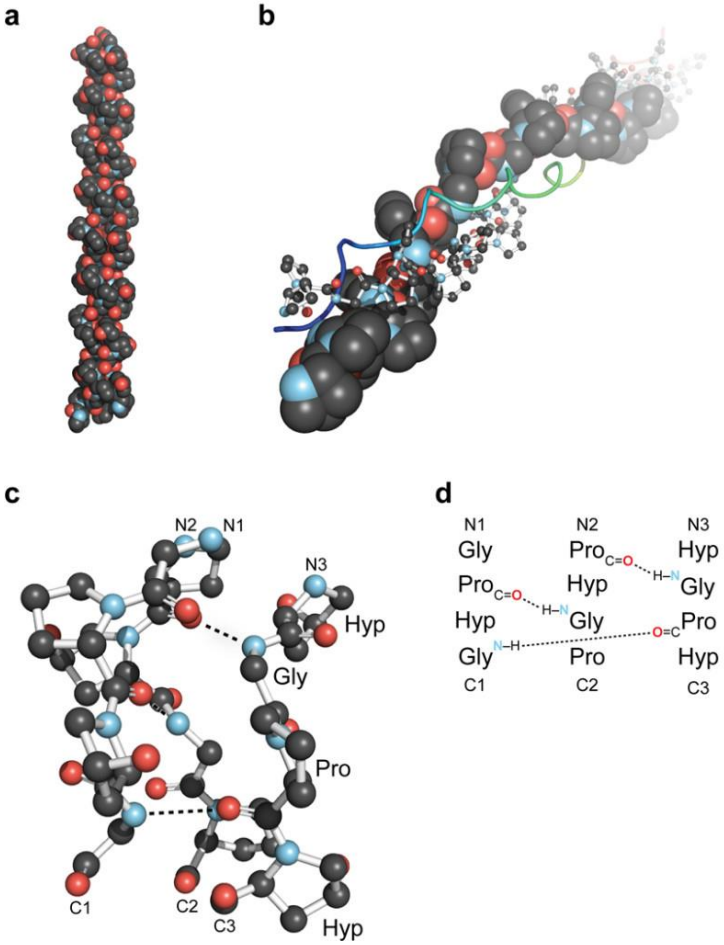


Figure 8: Overview of the collagen triple helix. (a) First high-resolution crystal structure of a collagen triple helix. (b) View down the axis of a (ProProGly) triple helix with the three strands depicted in space-filling, ball-and-stick, and ribbon representation. (c) Ball-and-stick image of a segment of collagen triple helix, highlighting the ladder of inter strand hydrogen bonds. (d) Stagger of the three strands in the segment in panel c [100].

Collagen pro-α chain is synthesized from a unique mRNA within the rough endoplasmic reticulum and is then transferred to the Golgi apparatus of the cell. During this transfer, some prolines and lysines residues are hydroxylated by the lysyl oxydase enzyme. Specific lysines are glycosylated and then pro-α-chains self-assemble into procollagen prior to their encapsulation in excretory vesicles. Following their passage through the plasma membrane, the propeptides are cleaved outside the cell to allow for the auto-polymerisation by telopeptides. This step marks the initiation of tropocollagen self-assembly into fibril and the agglomeration of fibril into collagen fibers. Fibril-forming collagens are the most commonly used in the production of collagen-based biomaterials [101].

2.7.2 Physical and Chemical Propetries

2.7.2.1 Natural Crosslinks

Mechanical and chemical stability derives from intra- and intermolecular crosslinks. Initially, the formation of crosslinks is mediated by lysyl oxidase during fibril formation [102].

The enzymatic activity is limited to the non-helical telopeptide regions and leads to the conversion of selective lysyl and hydroxylysyl residues to the corresponding aldehydes allysine and hydroxyallysine (Figure 9). While the fibrils associate, the aldehydes can spontaneously react. Intramolecular crosslinks form between two α-chains in the non-helical section of the same molecule by aldol condensation of two aldehydes [102], [103]. Intermolecular crosslinks occur between the telopeptide region of one collagen molecule and the helical region of a quarterly staggered, adjacent molecule. These bridges between two different tropocollagen molecules result from aldimine formation (non-, mono- or dihydroxylated dehydrolysinonorleucine (D-HLNL)) between aldehyde residues and ε-amino groups presented by lysine and hydroxylysine (Figure 9). The interchain bifunctional crosslinks are still reactive and continue to form polyfunctional crosslinks through multiple condensations with histidine, lysine, or hydroxylysine residues [104]. Besides the formation of enzymatic crosslinks there is a group of crosslinks derived from glycated lysine and hydroxylysine residues which occur more adventitiously [103]. Hence, through specific self-aggregation and crosslinking, collagen can form fibers of unusual strength and stability. The degree of crosslinking increases with age and stress [105] and consequently changes the properties of collagen material.

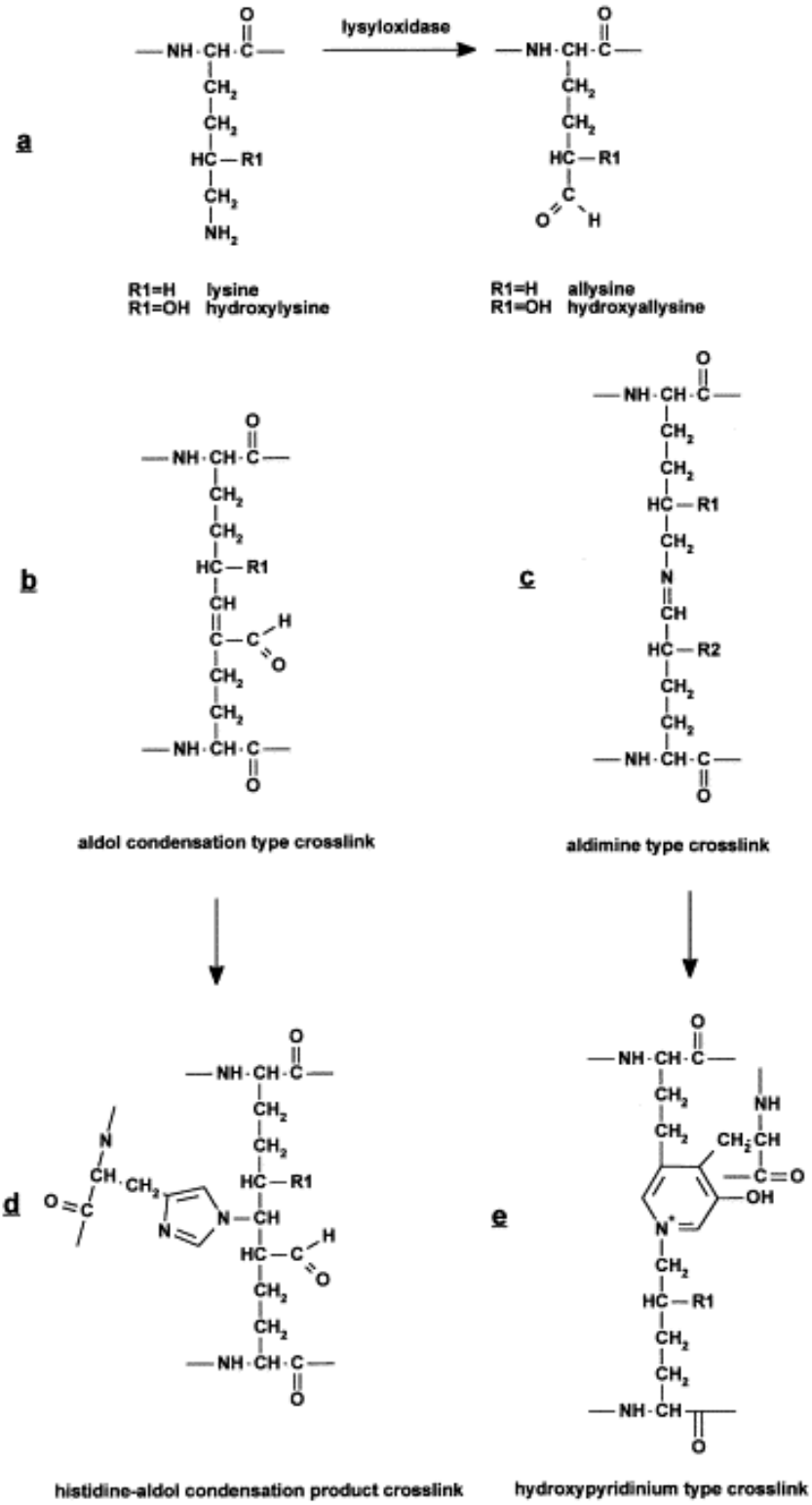


Figure 9: Chemical structures of natural collagen crosslinks. (a) Oxidation of lysine and hydroxylysine via lysyloxidase, (b) intramolecular aldol condensation type crosslink, (c) intermolecular aldimine (Schiffs' base) type crosslink (R1, R2=H, OH: dehydrolysinonorleucine, dehydrohydroxylysinonorleucine, dehydrodihydroxylysinonorleucine (may undergo Amadori rearrangement to from ketoamine), (d) condensation of aldol condensation type crosslink with hydroxyproline (can further condensate with lysyl residue to form histidinohydroxymerodesmosine) and (e) hydroxypyridinium type crosslink [96].

2.7.2.2 Degradation

Collagen is particularly resistant to attacks by neutral proteases. At neutral pH only specific collagenases cleave the native helix at a position, about threequarters of the way from the N-terminus [106]. Fibrils as aggregates of collagen molecules are degraded starting from the exterior.

Administration of exogenous collagen activates a complex cellular response which depends on the type of collagen material. Collagen degradation by all these cell types can occur either after phagocytosis of partially cleaved fibers or by extracellular proteases acting at either neutral or acid pH. Activation of macrophages has been shown to cause a pH decrease in the micro-environment of collagen implants to below pH 5 [107]. Together with the excretion of cathepsin from macrophages and neutrophils [108] this creates an acidic pathway for collagen breakdown.

In vitro degradation is usually simulated by incubation with bacterial collagenase, cathepsin, pepsin or trypsin [109].

2.7.2.3 Antigenicity and Immunogenicity

Selective removal of the non-helical component from the collagen molecule suppresses its antigenicity [110]. Additional chemical crosslinking, e. g. with glutaraldehyde reduces the antigenicity but does not eliminate it completely [111]. Thus, the immunogenic response depends on the collagen source as well as the test technique and the species used for animal experiments [112]. Despite theoretical concerns animal collagen in the form of sutures, hemostatic agents, and injectable collagen is considered safe [113] and only mildly antigenic [114], making it suitable for use as an implantable and injectable biomaterial.

2.7.2.4 Solubility for Isolation and Purification of Collagen

The major impediment to dissolution of collagen type I from tissue is the presence of covalent crosslinks between molecules. Collagen is insoluble in organic solvents. Watersoluble collagen represents only a small fraction of total collagen. The nature of the crosslinks prevalent in different tissues determines the particular solvent to be used and the corresponding yields.

The most commonly used solvents are neutral salt solution (0.15–2 M NaCl) or dilute acetic acid [115].

Dilute acidic solvents, e.g. 0.5 M acetic acid, citrate buffer, or hydrochloric acid pH 2–3 are more efficient than neutral salt solutions. The intermolecular crosslinks of the aldimine type are dissociated by the dilute acids and the repulsive repelling charges on the triple-helices lead to swelling of fibrillar structures [116].

It is possible to solubilize approximately 2 % of the tissue collagen with dilute salt or acid solutions. These collagen molecules can be reconstituted into large fibrils with similar properties as native fibrils by adjusting the pH or temperature of the solution [109].

The remaining 98 % is referred to as insoluble collagen although this dominant collagen material is not absolutely insoluble and can be further disintegrated without major damage to the triple-helical structures. The two most common approaches are the use of strong alkali or enzymes to cleave additional crosslinks and suspend or dissolve at first acid-insoluble structures.

Instead of disintegration and transfer into soluble material, extensively crosslinked collagen can be dispersed as opalescent, fine fibrillar suspensions by the use of mild denaturation agents and mechanical fragmentation usuallyat an acidic pH.

In additional steps collagen material can be subjected to chemical modifications such as succinylation [117], acetylation [118], methylation [119] or attachment to other polymers [120].

2.7.3 Crosslinking

In common with all natural polymers, one major problem with using collagen as the main constituent of a scaffold for orthopedic tissue engineering is that it has relatively poor mechanical properties. Natural crosslinking gives high tensile strength and proteolytic resistance to collagen. Dissociation of crosslinks can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from. Therefore, it is often necessary to confer mechanical firmness and collagenase resistance by introduction of exogeneous crosslinking into the molecular structure [121], [122].

Cross-linking of the collagen-based scaffolds is an effective method to modify the biodegrading rate (enzymatic resistence) and to optimize the mechanical property for implantation purposes. The principle of a cross-linking reaction relies on the modification of amine and carboxyl groups within the collagen molecules, to allow the formation of covalent bonds. Several methods have been developed to cross-link collagen scaffolds. These polymerization techniques are distributed among three types: physical, chemical and enzymatic crosslinking.

An alternative to covalent bond crosslinking is to promote the formation of ionic bonds between collagen molecules. This can be achieved by polycationic molecules such as chitosan, which create ionic bonds between its numerous amine groups and the carboxyl groups of collagen. These bonds are strong enough to stabilize the biomaterial structure and form a strong mechanical strength [123], [124]. The major advantage of this technique is to prepare the biomaterial in a one step process, where chitosan is mixed with collagen before freeze-drying, avoiding the need of further washing steps since chitosan is not toxic [125].

Enzymatic crosslinking agents like transglutaminase can be used to enhance tensile strength and enzymatic resistance of collagen-based biomaterial [126], [127], [128]. The major advantage with the approach of using a biologic polymerization technique is that no chemical residues or by-products remain in the scaffold structure, and therefore eliminate the risk of inducing cytotoxic effects.

A plethora of biomolecules can also be added to collagen solution to produce collagen-based biomaterials. These biomolecules, typically GAG, elastin and chitosan are added to the compound to potentially enhance the mechanical strength and to modulate cellular functions such as migration, proliferation and differentiation [129], [130], [131], [132], [133], [134], [135].

2.7.3.1 Physical Treatment

Alternative physical methods are pursued, including dry heat, exposure to ultraviolet or γ-irradiation, because chemical crosslinking agents is the potential toxic effect of residual molecules and/or compounds formed during in vivo degradation [136]. Collagen becomes partially denatured by these physical treatments [137]. In order to keep degradation

of the triple-helices to a minimum it is crucial for dehydrotermal (DHT) treatment to reduce the water content via vacuum as thoroughly as possible prior to heating [138]. Severe dehydration itself already induces amide formation and esterification between carboxy and free amino and hydroxyl groups, respectively [139], [140]. The combination of degradation and crosslinking allows non-specific enzymes to attack and solubilize fragments of the crosslinked material. Formation of crosslinks during UV-irradiation is thought to be initiated by free radicals formed on aromatic amino acid residues [137] which indicates a rather limited maximum degree of crosslinking due to the small number of tyrosine and phenylalanine residues in collagen.

2.7.3.2 Syntetic Crosslinkers

Due to the large number of functional side groups it contains, collagen readily undergoes chemical crosslinking. This is usually performed using bifunctional reagents as glutaraldehyde (GTA), dialdehydes and diisocyanates, as well as carbodiimides, diepoxides, polyepoxy compounds, formaldehyde and acyl azide methods [141], [142], [143], [144].

Using this agents it is possible to create a three-dimensional network that results in collagen scaffolds with enhanced mechanical properties. However, glutaraldehyde and 1,6-hexamethylene diisocyanate have been found to be cytotoxic and as such carbodiimide or acyl azide methods have been introduced [145], [146]. However, both methods have limited cross-linking ability due to their short length structure and inability to polymerise [147].

Carbodiimides

Crosslinking with carbodiimides, especially 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) offers the main advantage over aldehydes, HDC, or epoxy compounds in that these carbodiimides only facilitate the formation of amide bonds between carboxylic and amino groups on the collagen molecules without becoming part of the actual linkage. Thus, bifunctional crosslinking agents are obviated. Carbodiimides first couple to a carboxylic group to form *o*-isoacylurea structures. The resulting activated intermediate is attacked by a nucleophilic primary amino group to form an amide crosslink and the isoureaderivative of the applied carbodiimide is eliminated and can be washed out [148], [149]. The susceptibility of carbodiimide crosslinked material to enzymatic degradation can be controlled by varying the degree of cross-linking via the reaction conditions [150].

Nowadays it si most commonly used *N*-ethyl-*N*′-[3–dimethylaminopropyl]carbodiimide/ *N*-hydroxy succinimide (EDC/NHS) as a non-toxic cross-linker which crosslinks without incorporation of the crosslinking reagent [82]. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) or acylazide were used to generate peptide-like bonds in biomaterials [151]. Use of EDC and *N*-hydroxysuccinimide (NHS) to crosslink collagen seems to yield biomaterials with good biocompatibility, higher cellular differentiation potential and with increased resistance against enzymatic degradation [152].

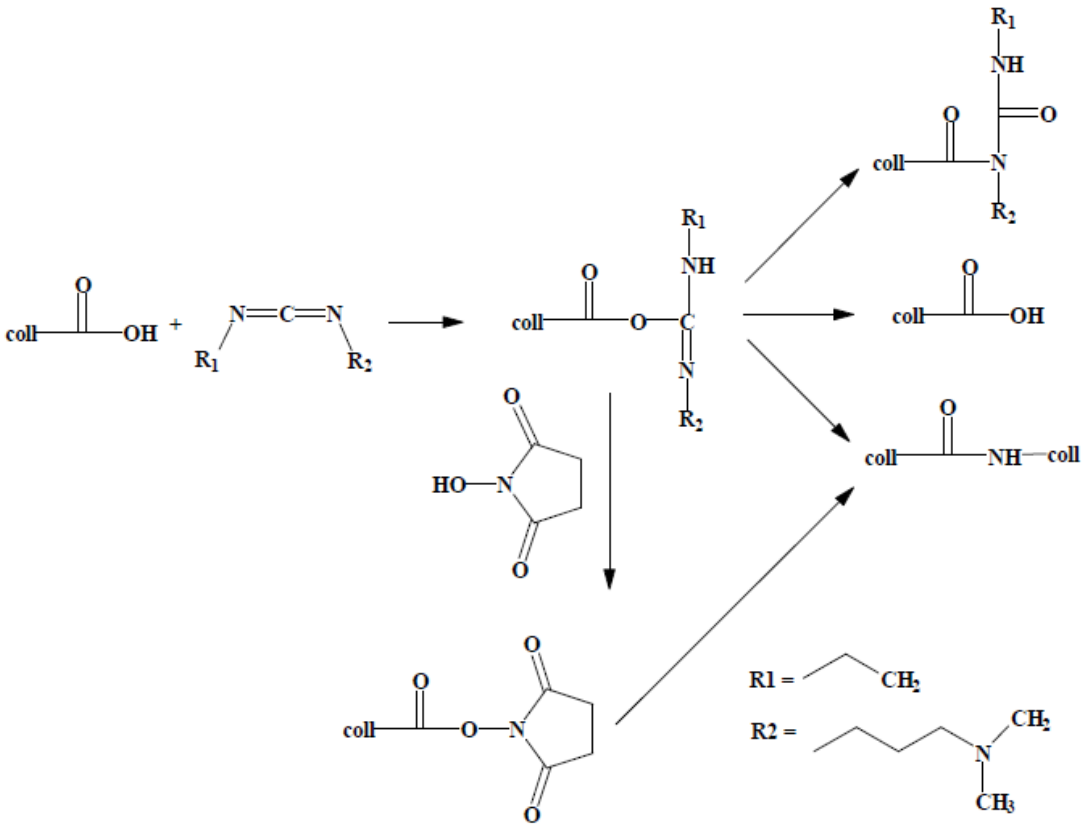


Figure 10: Schematic reaction of collagen with EDC/NHS.

2.7.3.3 Natural Cross-linking Agents

The syntetic substances for cross-linking are tried to be replaced by crosslinking agent able to form stable and biocompatible crosslinked products, without added cytotoxicity problems. This is the reason of the increasing demand for natural crosslinking agents for medical applications [153]. Natural crosslinkers have high biological activity and can significantly improve collagens properties while preserving its triple-helical structure [154].

Recent data demonstrate that plant extracts not only optimally stabilise collagen scaffolds and bring about mechanical properties similar to native extracellular matrix assemblies, but also do not compromise cell attachment, proliferation and growth [155], [156], [157], [158], [159], [160], [161], [162], [163].

Genipin is a natural cross-linking agent found in plants, which has been shown to be quite efficient in protein cross-linking [164]. Using citric acid as a crosslinked molecule will avoid inducing toxic substances. CA can easily react with the amidogen on the collagen chain and form cross-links to enhance the mechanical strength [165]. Stabilisation using M. rubra could be a valuable alternative to aldehyde approaches for the construction of three-dimensional scaffolds that would imitate native extracellular matrix assemblies [166]. The interactions between protein and polyphenol can involve hydrogen bond, covalent linkage, ionic and hydrophobic bonding. The hydrogen bond interaction is mainly responsible for the stabilization of collagen by procyanidin. The procyanidin treatment does not destroy the triple helix conformation of collagen, but induces the aggregation of collagen microfibrils [167].

2.8 Scaffold Fabrication Techniques

In the last decade, various fabrication methods for construction of three-dimensional biomimetic scaffolds, including electrospinning [168], [169], [170], phase-separation [171], [172], freeze drying [173], [174] and self-assembly [175], [176] have been developed for tissue engineering and regenerative medicine. These scaffolds can mimic the architecture of the native extracellular matrix at the nanoscale level (eg, hierarchical architecture formed with nanofibers and nanopores), which provides the initial space for regeneration of new tissue [177].

In addition, surface modification methods (eg, plasma exposure) used for nanostructured scaffolds can introduce functional groups (e. g. Arg-Gly-Asp peptide) onto the surface of the scaffolds, which directly and significantly enhance cell attachment, migration, and proliferation.

Traditional tissue engineering methods use a "top-down" approach, in which cells are seeded onto a scaffold with biocompatible and biodegradable properties, and are expected to populate in the scaffold and create their own extracellular matrix. Despite several thin or avascular tissues, such as skin [178], bladder [179], and cartilage [180], having been engineered successfully via the top-down approach based on these biomimetic scaffolds in vitro, the fabrication of complex larger functional tissues (eg, liver and kidney) with high cell densities and high metabolic requirements still faces challenges. This is mainly because of the limited diffusion properties of biomimetic scaffolds [181].

Tissue engineered constructs can also be fabricated by the assembly of smaller building blocks. This approach mimics much of the native biology that is often made from repeating functional units. For example, in the liver, the sinusoid is the repeating functional unit. Bottom-up approaches can be used to generate functional units that can be assembled in a modular approach to generate larger scaffolds [182]. Fabrication of tissue building blocks can be achieved via multiple approaches, including fabrication of cell-encapsulating microscale hydrogels (microgels), self-assembled cell aggregation, generation of cell sheets, and direct printing of cells [183], [184], [185] (Figure 11). These microscale building blocks can be successfully assembled into complex tissue constructs, with control over features such as the shape and composition of individual blocks [186], [187].

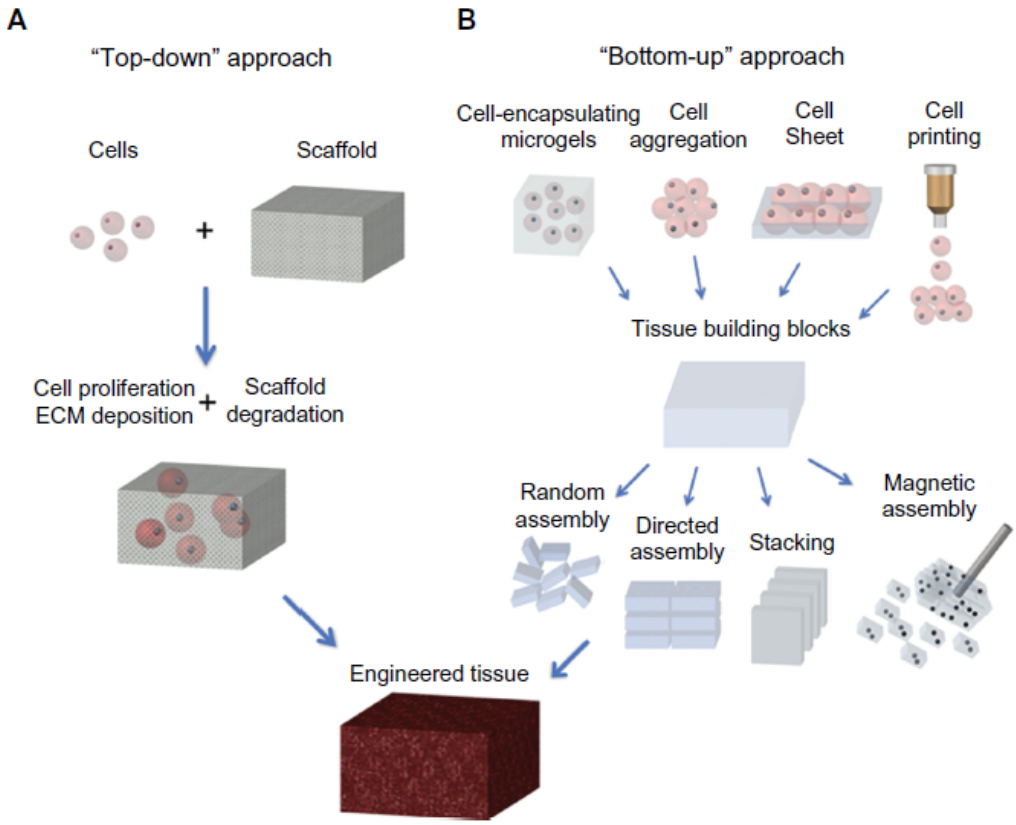


Figure 11: Schematic of "top-down" and "bottom-up" approaches for tissue engineering. (A) In the top-down approach, cells are seeded on a biocompatible and biodegradable scaffold and are expected to populate in the scaffold and create their own extracellular matrix. (B) In the bottom-up approach, various methods are utilized for generating tissue building blocks and these units can be engineered into large tissue constructs via multiple assembling methods [188].

2.8.1 Conventional Scaffold Fabrication Techniques

Several techniques have been developed to process synthetic and natural scaffold materials into porous structures. These conventional scaffold fabrication techniques are defined herein as processes that create scaffolds having a continuous, uninterrupted pore structure which lacks any long-range channelling microarchitecture.

Conventional scaffold fabrication techniques include:

- fiber bonding [189] and [190],
- phase separation [191] and [192],
- solvent casting/particulate leaching [193], [194], [195] and [196],
- membrane lamination [197],
- melt molding [198],
- gas foaming/high pressure processing [199] and [200],
- hydrocarbon templating [201],
- freeze drying [202], [203],
- combinations of these techniques (e. g., gas foaming/ particulate leaching [204], etc).

Although conventionally produced scaffolds hold great promise and have been applied to engineer a variety of tissues with varying success, most are limited by some forms of flaws, which restrict their scope of applications.

Table 2: Summarizes the advantages and limitations of these conventi onal techniques [205].

Technique	Advantages	Limitations
Fiber bonding	Easy process High porosity High surface area to volume ratio	High processing temperature for non-amorphous polymer Limit range of polymers Lack of mechanical strength Problems with residual solvent Lack of control over micro-architecture
Phase separation	Allows incorporation of bioactive agents Highly porous structures	Lack of control over micro-architecture Problems with residual solvent Limited range of pore sizes
Solvent casting and particulate leaching	Highly porous structures Large range of pore sizes Independent control of porosity and pore size Crystallinity can be tailored	Limited membrane thickness Lack of mechanical strength Problems with residual solvent Residual porogens
Membrane lamination	Macro shape control Independent control of porosity and pore size	Lack of mechanical strength Problems with residual solvent Tedious and time-consuming Limited interconnected pores
Melt moulding	Independent control of porosity and pore size Macro shape control	High processing temperature for non-amorphous polymer Residual porogens
Polymer/ceramic fiber composite-foam	Good compressive strength Independent control of porosity and pore size	Problems with residual solvent Residual porogens
High-pressure processing	Organic solvent free Allows incorporation of bioactive agents	Nonporous external surface Closed pore structure
High-pressure processing and particulate leaching	Organic solvent free Allows incorporation of bioactive agents Highly porous structures Large range of pore sizes Independent control of porosity and pore size	Limited interconnected pores Lack of mechanical strength Residual porogens
Freeze drying	Highly porous structures High pore interconnectivity	Limited to small pore sizes
Hydrocarbon templating	No thickness limitation Independent control of porosity and pore size	Problems with residual solvent Residual porogens

2.8.2 Solid Freeform Fabrication Techniques

Solid freeform fabrication (SFF) techniques are computerized fabrication techniques that can rapidly produce highly complex three-dimensional physical objects using data generated by computer-aidede design (CAD) systems, computer-based medical imaging modalities, digitizers and other data makers [206]. Unlike conventional computerized machining processes which involve the removal of materials from a stock, SFF techniques uses the underlying concept of layered manufacturing [206], [207] whereby three-dimensional objects are fabricated with layer-by-layer building via the processing of solid sheet, liquid or powder material stocks.

Solid freeform fabrication (SFF) may produce scaffolds with customised external shape and predefined and reproducible internal morphology, which not only can control pore size, porosity an pore distribution, but can also make structures to increase the mass transport of oxygen and nutrients throughout the scaffold.

The flexibility and outstanding manufacturing capabilities of SFF have been employed for biomedical applications ranging from the production of scale replicas of human bones [208] and body organs [209] to advanced customized drug delivery devices [210] and other areas of medical sciences including anthropology [211], palaeontology [212] and medical forensics [213].

SFF include:

- three dimensional printing (3DP),
- stereolithography (SLA),
- fused deposition modelling (FDM),
- selective laser sintering (SLS),
- 3D plotter,
- phase-change jet printing [214].

Table 3: Summarizes the capabilities and limitaions of the three solid free-form fabrication techniques [205].

Technique	Build resolution, [mm]	Advantages	Limitations
3D-P	0,100 ± 0,020 Achievable pore size = 45–100 μm	Easy process High porosity High surface area to volume ratio Complete pore interconnectivity Macro shape control Independent control of porosity and pore size Wide range of materials	Use of toxic organic solvents Lack of mechanical strength Limited to small pore sizes
FDM	0,050 ± 0,127 Achievable pore size = 250–1000 μm	High porosity High surface area to volume ratio Complete pore interconnectivity Macro shape control Independent control of porosity and pore size Good compressive strengths Solvent free	High processing temperatures Limited material range Inconsistent pore opening in x-,y- and z-directions Pore occlusion at boundaries Requires support structures for irregular shapes
SLS	0,076 ± 0,250 Achievable pore size = 45–100 μm	High porosity High surface area to volume ratio Complete pore interconnectivity Macro shape control Independent control of porosity and pore size Good compressive strengths Wide range of materials Solvent free	High processing temperatures Limited to small pore sizes

2.8.3 Collagen Scaffold the Most Used Fabrication Techniques

Many extracellular proteins, including collagen, have a nanoscale fibrous structure (50–500 nm in diameter) in vivo which has been found to enhance cell attachment, proliferation, and differentiation [215], [216]. Nanofibrous biomimetic scaffolds consist of biodegradable polymer nanofibers, which can be fabricated by several methods, including electrospinning, phase-separation, and self-assembly, and can mimic the nanofibrillar structure of the extracellular matrix in vivo (Figure 12). In Table 4 are summarized parameters of mentioned methods.

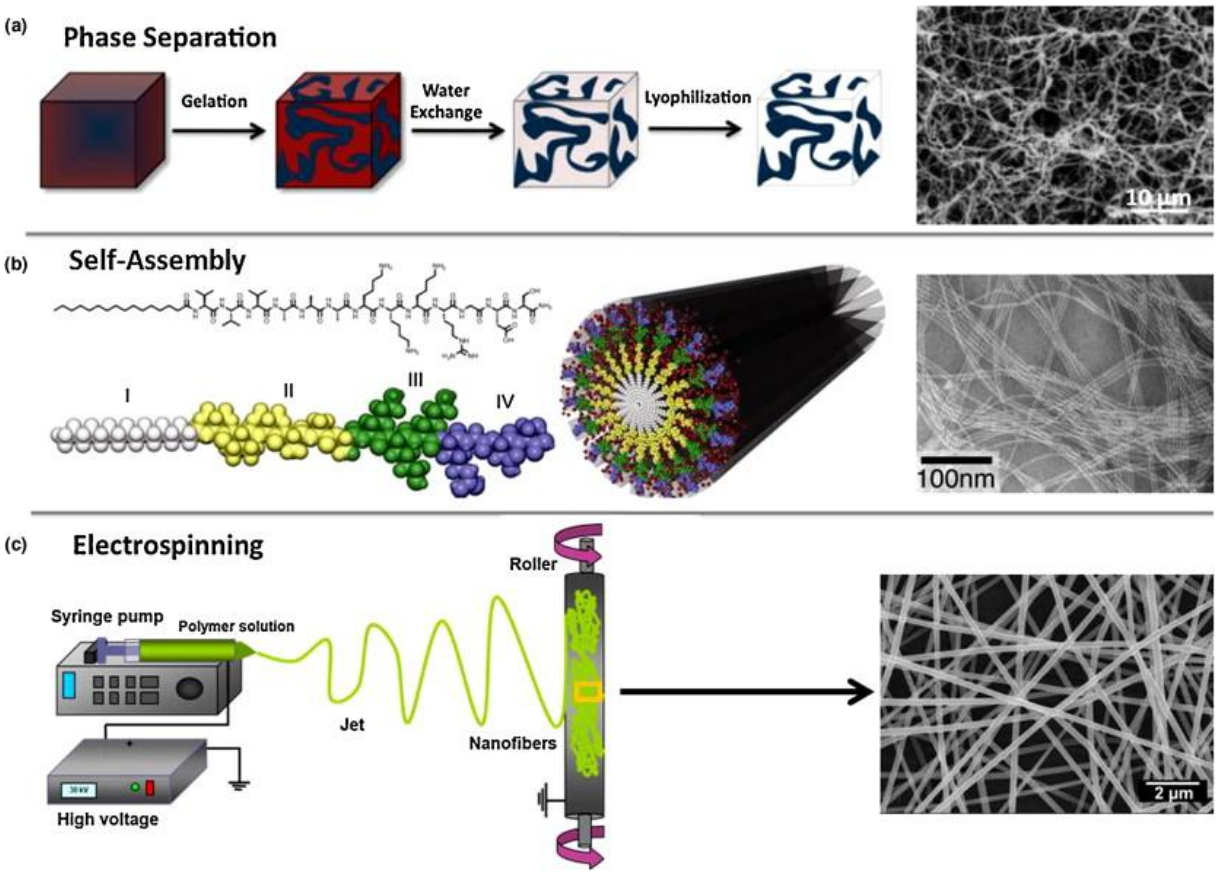


Figure 12: Schematic of current techniques (a) phase separation, (b) self-assembly, and (c) electrospinning to create fibrillar structures in synthetic scaffolds [217].

Table 4: Different methods for fabricating three-dimensional nanostructured scaffolds [188].

Method	Polymer	Diameter of fiber [nm]	Porosity	Cell viability	Material types/ commercially available	Applications for TE
Electro-spinning	PLLA PLGA PGA/PLLA Collagen/PCL Chitosan/PCL Silk/HAP	400–1100	80%–95%	<80%	30/ available	Skin Cartilage Vascular Nerve Bone
Phase-separation	PLLA/HAP PLGA Chitosan	50–500	60%–98%	<98%	15–20/ available	Bone
Freeze-drying	Chitosa/PLLA Collagen/GAG Gelatin-PHEMA	50–450	30%–80%	<90%	15/ available	Tendon Bone/tendon Skin
Self-assembly	Peptide	5–300	80%–90%	60%–95%	5–10/ not known	Cartilage

Here, we focus only on the method we use for fabrication porous collagen sponges – phase separation, especially freeze-drying fabrication method.

2.8.3.1 Phase Separation

The phase separation process can be induced thermally or by a nonsolvent and was developed for the fabrication of 3D porous scaffolds [174], [218], [219]. Induction of the phase separation process using a nonsolvent commonly results in scaffolds with a heterogeneous pore structure which is not suitable for fabrication of tissue engineering

scaffolds, which generally need a uniform pore structure [220]. The thermally induced phase separation process takes place when a homogeneous polymer solution becomes thermodynamically unstable under certain temperature conditions and tends to separate into a multiphase system domains, comprising a polymer-lean phase (with a low polymer concentration) and a polymer-rich phase (with a high polymer concentration) [221], [222]. Subsequently, the polymer-rich phase solidifies to form a matrix while the polymer-lean phase turns into pores as a result of solvent removal. Thermally induced phase separation can be divided into solid-liquid phase separation and liquid-liquid phase separation.

The solid-liquid phase separation proces, also called emulsion freeze drying, is used to induce solvent crystallization from a polymer solution by lowering the temperature, which leads to formation of pores after removal of solvent crystals.

Freeze-drying has emerged as a drying process for converting solutions of labile materials into solids of sufficient stability for distribution and storage in applications such as food science, pharmaceuticals, and enzyme stabilization [223], [224], [225]. Freeze-drying involves three major steps: the solution is frozen at a low temperature (−70 °C to −80 °C); the frozen sample is located in a chamber in which the pressure is lowered (to a few millibars) through a partial vacuum, known as the primary drying process, in which ice in the material is removed by direct sublimation; and most of the unfrozen water in the material is removed by desorption in a secondary drying process.

Porosity and pore diameter in porous scaffolds prepared using the freeze-drying method can be regulated by the freeze-drying pressure [219], (Figure 13). Although there are several advantages of the freeze-drying method, including use of water and ice crystals instead of an organic solvent in the scaffold fabrication process, which is more suitable for biomedical applications, it is still a big challenge to engineer scaffolds with hierarchical structures (e.g. vascularized systems) using this approach.

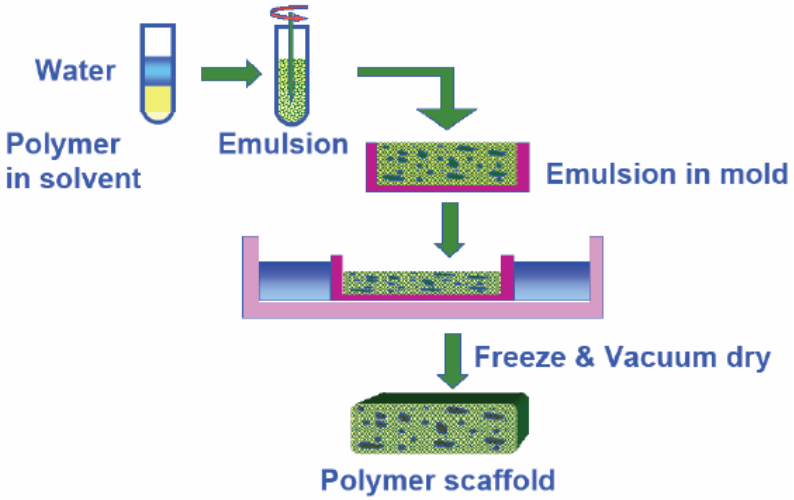


Figure 13: The schematic diagram of solid-liquid phase separation – freeze-drying technique [226].

In the liquid-liquid phase separation process, polymer solutions with an upper critical temperature form a bicontinuous structure (both polymer-lean phase and polymer-rich phase) [4], [227].

Various synthetic polymeric nanofibers have been fabricated using phase separation methods for tissue engineering applications [171], [172], [192], [228]. Several natural polymers have been fabricated into three-dimensional porous scaffolds, for instance, chitosan scaffolds with nanofibrous or microfibrous structures prepared by solid-liquid phase separation.

The porosity and fiber size of the scaffolds can be regulated by several methods, including phase separation temperature and polymer concentration [229].

Phase separation holds great potential for fabrication of three-dimensional nanofibrous scaffolds with uniform pore structures through dual or multiple phase separation processes. In addition, phase separation can engineer three-dimensional shapes via several techniques, including solid free-form fabrication, rapid prototyping, and computer-assisted design and manufacture. However, limitations such as limited material selection and inadequate resolution still exist.

2.9 Collagen-Based Scaffolds for Tissue Engineering and Their Application

The material of scaffolds for tissue engineering can be any biomaterial that mimics one or multiple characteristics of the natural ECM [230], but is expected to function as a scaffold to replace natural collagen-based ECM. Much research has been reported on collagen, its denatured forms or collagen-based materials as biomaterials for scaffold fabrication in tissue engineering [231], [232], [233], [234], [235], [236].

2.9.1 Pure Collagen Scaffold

Collagen type I, II and III could form collagen fibers that are similar to or even fully reproduced the structural and biological properties of the natural collagen ECM under optimizing conditions. By electrospinning, collagen type I produced fibers exhibiting the 67 nm D-repeat banding pattern, which is a characteristic of native collagen [237].Using lyophilization can be determined the collagen concentration in solutions which determines the mechanical properties of the scaffold [238]. Proper concentration could be chosen according to the implant position of the scaffold.

Despite the excellent biological properties of the pure collagen scaffold, it presents poor mechanical properties and structural stability. Physical treatment or chemical agents can be used to achieve intermolecular cross-linking of collagen, thus modifying the properties of the collagen scaffold.

2.9.2 Collagen/Natural Polymer Blend Scaffold

Cross-linking strategies of the pure collagen scaffold enhance the mechanical and structural properties, but may introduce negative effects on cellular response in vivo. Hence, a mixture of natural or synthetic polymers can be used to overcome the limitations of the monocomponent system. Natural polymers (such as chitosan, silk fibroin, hyaluronic acid, alginate, etc.) have been widely used in tissue engineering due to their similar features to native ECM [239].

2.9.3 Collagen/Synthetic Polymer Blend Scaffold

Blending of collagen with synthetic polymers also makes it possible for scaffolds to perform both with optimal mechanical and biological properties in specific engineering applications. In this case, the synthetic polymer undertakes mechanical support to the structure of scaffolds, while collagen on the surface and inside of the scaffolds provides

cell recognition signals, which is crucial for cell behaviors and development [240]. Scaffolds composed of collagen and synthetic polymers, such as poly (ε-caprolactone) (PCL), polylactic acid (PLA), poly(ethylene glycol) (PEG), polyglycolide (PGA), poly(lactide-*co*-glycolide) (PLGA) and polyvinyl alcohol (PVA), have been widely used for tissue engineering.

2.9.4 Collagen/Inorganic Hybrid Scaffold

Organic-inorganic composite materials have drawn much attention due to their ability to combine excellent properties of individual constituents. Hybridization can achieve tailor-made performances (such as morphology, stiffness, degradation) and meet various requirements in tissue engineering [241], [242]. Several inorganic materials, such as hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), silicate and β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$), have been used in the construction of tissue engineering scaffolds.

2.9.5 Collagen Scaffold Modified with Growth Factors

Growth factors can regulate a variety of cellular processes; they are intercellular signaling molecules promoting cell migration, proliferation, differentiation and maturation depending on their type [243].

Generally, there is an initial burst release of growth factors encapsulated in the scaffold, which is usually not effective and welcome. Hence, an appropriate loading method is crucial to the stable, sustainable and controllable release of growth factors on the scaffold.

2.9.6 Applications of Collagen-Based Scaffolds in Tissue Engineering

In this section, several typical applications of collagen-based scaffolds in tissue engineering will be introduced, respectively.

2.9.6.1 Nerve Tissue

Collagen-based biomaterials have been extensively studied as a promising nerve guide [244], [245], [246], [247]. Multiple compositions of collagen-based nerve conduits have recently been tested with positive results compared to clinically used autografts. Most collagen nerve guides are engineered from crosslinked collagen solution molded into tubular shape [248]. Pore orientation [249], [250], addition of neurotrophic factors [251], [252] and cell delivery [253], [254], [255] are currently being investigated in an attempt to enhanced nerve guides for clinical applications. Collagen-based biomaterials can also be used to develop innovative three-dimensional tissue-engineered nervous system models to promote 3D axonal migration and myelination of sensory or motor neurons by Schwann cells through a connective tissue [256].

2.9.6.2 Bone/Cartilage Tissue

Bone tissues are mainly composed of collagen type I and HA with a small amount of type V. Hence, collagens blended with nano-inorganic materials are more widely used to prepare scaffolds that mimic natural ECM of bone in bone repair.

Cartilage defects cause joint pain and loss of mobility [257]. Chondrocytes show low rates of regeneration due to their non-mobility and the absence of progenitor cells and vascular networks in the tissue [258]. MSCs have been commonly employed as a main source of seeding cells in cartilage tissue engineering.

2.9.6.3 Tendon/Ligament Tissue

Tendons and ligaments are fibrous connective tissues, with collagen comprising 70 % to 80 % of their dry weight [259]. Both tendon and ligament have weak spontaneous regeneration ability and never totally recover from full-thickness lesions. The substantial donor site morbidity limits autograft applications for injured tissue and encourages the search for alternative solutions. Collagen scaffold provides an excellent way for tendon/ligament repair and regeneration.

2.9.6.4 Vascular Disseases

Two main problems arise in the domain of vascular diseases: cardiovascular malfunction and venous or arterial pathologies such as atherosclerosis. In the case of heart diseases, tissue engineering solutions rely principally on acellular matrix colonization and implantation due to the complex structural architecture of the heart like heart valves [260], [261].

Vascular grafts may induce immediate thrombus after implantation due to their lack of healthy endothelium. Vascular endothelialization can reduce thrombosis, inhibit excessive hyperplasia of intima and significantly improve the long-term patency rate of artificial blood vessels.

Considering the typical requirement of autologous cells, whose amplification in vitro is time consuming, cell-free vascular grafts have gained much attention.

Combining the cell-free vascular graft with endogenous ECs represents a great progress in vascular tissue engineering. Future research directions may be focused on the development of vascular material and immobilization of growth factor.

2.9.6.5 Skin and Cornea

Skin and cornea share a similar tissue structure: dermis and stroma both being connective tissues; epidermis and cornea being stratified epithelia. Collagen-based wound dressings have been applied for decades for burn coverage applications and ulcer treatment [262], [263]. Highly sophisticated and innovative tissue-engineered skin models have been developed with melanocytes [264], a capillarylike network [265], dendritic cells [266], sensory innervation [133], [256], adipose tissue [267], and tissue reproducing psoriatic or sclerotic phenotypes [268], [269].

Mesenchymal stem cell delivery to the wound bed in collagen-based biomaterial is a growing topic in wound healing [267], [270], [271]. The combination of collagenous biomaterials and stem cells could also be a valuable strategy to treat corneal defects. In the last decade, collagen scaffolds have been intensively studied for the delivery of limbal epithelial stem cells to damaged cornea [272], [273], [274], [275], [276], [277]. Advances in collagen-based corneal scaffolds also include the utilization of recombinant human collagen [276], [278], [279], [280], [281], the secretion of collagen by the fibroblasts themselves (self-assembled fibroblasts sheets) [282] and surface modification to reduce extensive endothelialization [283].

Many organic/inorganic materials can be used to strengthen the properties of collagen scaffolds in scientific research, but the materials approved by the U.S. Food and Drug Administration (FDA) for clinical applications are limited. The FDA has approved only collagen, hyaluronic acid, PLLA, HA and non-biodegradable polymethylmethacrylate (PMMA) beads used as dermal fillers since July 2015 [284].

2.9.6.6 Urogenital System

The use of collagen-based biomaterials in the domain of urogenital diseases and dysfunctions rely principally on acellular ECM from either small intestinal submucosa (SIS) or bladder submucosa (BSM) [285], [286]. More recent surgical procedures aiming to solve genitourinary disorders use acellular collagen scaffolds in bladder augmentation [287], [288], [289] and urethral stricture [291], [292], [293].

Collagen-composite scaffolds populated with the patient's own urothelial and muscle cells or self-assembled fibroblast sheets are also a promising strategy for bladder augmentation [294], [295], [296]. Vesico-urethral reflux and incontinence are other defects of the urogenital system which can also be solved using injection of collagen biomaterials [297], [298], [299].

2.9.6.7 Dermal Filler, Wound Dressing and Delivery Systems

FDA approved dermal filler commonly used in facial rejuvenation or reconstructive surgery is using collagen from three distinct sources: Bovine Zyderm®, porcine Evolence™, human CosmoDerm® and Cymetra® [300], [301]. Although other collagen-based biomaterials are available for this purpose [302], these products can be useful for medical office-based interventions.

Wound dressings that are also delivery systems represent an interesting application for collagenbased applications.

The delivery properties of collagen-based biomaterials also display great potential for ulcer treatment [250]; abdominal wall defect reconstruction [303], [304], [305]; implants delivering antibiotics [306], [307], [308], [309]; gene therapy delivered by collagen matrix [310].

Collagen scaffolds have also shown to accurately deliver cells, proteins, drugs and nucleic acids on a predictable and long-term basis [311], [312], [313], [314]. The biodegradability of collagen and its low immunogenicity make it a substrate of choice for internal and topical pharmacogenomical applications.

3 EXPERIMENTAL PART

3.1 Chemicals

- Collagen – Type I collagen, 8% aqueous solution, Výzkumný ústav pletařský a.s., Czech Republic, was freeze-dried to obtain 100% collagen
- Bovine platelet lysate – VA-Bios s.r.o. company, Brno, Czech Republic
- *N*-(3-Dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride (EDC) – Sigma Aldrich Chemical Company, Germany, was diluted to obtain 50 $mmol \cdot dm^{-3}$ solution in 98% ethanol (w/w)
- *N*-hydroxysuccinimide (NHS) – Sigma Aldrich Chemical Company, Germany, was diluted to obtain 25 $mmol \cdot dm^{-3}$ solution in 98% ethanol (w/w)
- Calcium salt of oxidized cellulose – BLOODCARE powder, LIFE LINE plus s.r.o.
- $Na_2HPO_4 \cdot 12\ H_2O$ – Lachema a.s., Czech Republic
- Chitin/chitosan-glucan complex prepared by Abdel-Lattif Abdel-Mohsen (CEITEC BUT, Brno, CZ).
- Chitosan – Chitosan from crab shells – Sigma Aldrich, highly viscous, 2-Amino-2-deoxy-(1→4)-β-D-glucopyranan, Poly-(1,4-β-D-glucopyranosamin)
- Sodium hydroxide – Sigma-Aldrich, Germany
- Acetic acid – Sigma-Aldrich, Germany
- Ethanol – Sigma-Aldrich, Germany
- Hydrochloric acid – Sigma-Aldrich, Germany
- Isopropyl alcohol – Sigma-Aldrich, Germany
- Ultrapure water (type II according to ISO 3696) was prepared on our Elix 5UV Water Purification System (Merck spol. s.r.o.)

3.2 Equipments

- Desintegrator – IKA Ultra Turrax ® T18 basic
- Centrifuge – Med. Instruments, MPW-350R
- Lyophilizator – ALPHA 2-4 LSC, Chaist ®
- FTIR-ATR analyzer – Tensor 27, Bruker
- Analytical balances – DENVER INSTRUMENT, SI-234A
- Scanning electron microscope – Tescan, Lyra3 XM
- DSC analyser – TA Instruments, DSC Q2000

3.3 Preparation of Chitin/Chitosan-Glucan Complex

Mycelium was produced from schizophyllum commune strain as a source for extraction of chitin/chitosan-glucan complex from the collection of microorganism of biotechnological laboratories of Contipro Biotech Ltd., Czech Republic.

Chitin/chitosan-glucan complex was kindly prepared by Dr. Abdel-Lattif Abdel-Mohsen in two steps procedure. Firstly, dry mycelium was fermented from schizophyllum commune (S. commune) and treated with sodium hydroxide. Cake of mycelium was filtered off and the insoluble part was collected as chitin-glucan complex. Secondly, mixture of chitin-glucan complex was dispersed in different concentration of sodium and heated up for 5 h. The product was filtered and washed with distillated water until neutral pH, dried at 50 °C. The alkali insoluble material of chitin/chitosan-glucan complex yielded 72 percent.

3.4 Preparation of Samples

Samples were prepared in two series to compare the effect of crosslinking on the properties of the scaffolds. Table 5 summarizes the weight percent of used polymers in final scaffold, modification by PRP and whether samples were exposed to the crosslinking agent (EDC/NHS).

Table 5: Composition of prepared collagen sponges

Serie	Scaffold	$w_{collagen}$, [%]	$w_{additive}$, [%]	w_{PRP}, [%]	EDC/NHS
1	Collagen	0.5	×	×	Yes
	Collagen/PRP	0.5	×	10	Yes
	Collagen/CaOC	0.5	0.5	×	×
	Collagen/CaOC/PRP	0.5	0.5	10	×
	Collagen/Chitosan	0.5	0.5	×	Yes
	Collagen/Chitosan/PRP	0.5	0.5	10	Yes
	Collagen/Complex	0.5	0.5	×	×
	Collagen/Complex/PRP	0.5	0.5	10	×
2	Collagen	0.5	×	×	Yes
	Collagen/PRP	0.5	×	10	Yes
	Collagen/CaOC	0.5	0.5	×	Yes
	Collagen/CaOC/PRP	0.5	0.5	10	Yes
	Collagen/Chitosan	0.5	0.5	×	Yes
	Collagen/Chitosan/PRP	0.5	0.5	10	Yes
	Collagen/Complex	0.5	0.5	×	Yes
	Collagen/Complex/PRP	0.5	0.5	10	Yes

3.4.1 Collagen Sponges

Collagen solutions with concentration of 1 % (*w*/*w*) were prepared from lyophilized collagen in ultrapure water by desintegrating the swelled collagen fibers using IKA desintegrator at 8 000 rpm in ice bath. The solutions were replaced to tubes and centrifuged for 1 min at 1000 rpm at 4 °C to remove air bubbles. The adequate volume of homogenized solutions was poured into the holes of well-plate and freeze-dried.

3.4.2 Collagen Sponges with CaOC

Adequate weight of calcium oxidized cellulose was weighed on analytic balance and put into ultrapure water and leave for 1 hour to be stiring in fridge. The homogenous solution of CaOC was dropply poured into the solution with concentration weight of 1 % collagen and mixed by desintegrator. The adequate volume of homogenized solutions was poured into the holes of well-plate and freeze-dried. The final concentration of collagen and CaOC was 0.5 % (*w*/*w*).

3.4.3 Collagen Sponges with Chitosan

Collagen sponges with chitosan were prepared by the same procedure as the collagen sponges with CaOC.

3.4.4 Collagen Sponges with Complex

Collagen sponges with complex were prepared by the same procedure as the collagen sponges with CaOC.

3.4.5 Crosslinking of Collagen Sponges with Carbodiimides

Crosslinking agent with carbodiimides – *N*-ethyl-*N′*-[3-dimethylaminopropyl]carboimide/ *N*-hydroxy succinimide (EDC/NHS) was prepared at appropriate concentration (50 nM/25 nM) in the volumetric flask by mixing and used for collagen scaffolds crosslinking.

After crosslinking process, crosslinked scaffolds were twice with 0.1 M Na_2HPO_4 solution and then 3 times with water for removal of byproducts. Final products were freeze-dried again.

3.4.6 Sponges Enriched with PRP

Sample enrichment with PRP was performed after lyophilization of biopolymer mixture and crosslinking if performed. PRP water solution of 0.1 % was poured onto the sponge and relyophilized again.

3.5 Characterization of the samples

3.5.1 Morphology and Porosity

The morphology and microstructure of lyophilized collagen scaffolds were studied using scanning electron microscopy (SEM). For better resolution, samples were coated with the 20 nm of gold layer. All observations were made in the secondary electron emission mode at 5 kV acceleration voltage that due to very specimen sensitive such as scaffolds is used, since at higher voltages it could degrade.

Average pore size of scaffolds was characterized from the SEM vizualization using image analyses program ImageJ. One measurement consisted of 200 mesured values in image with resolution of 4 mm. There was also evaluated the porosity by this program.

3.5.2 Swelling Ratio and Water Content

The stability of the sponges at physiological temperature with respect to physical, chemical, and biological activity is to be assessed.

Each porous scaffold was cut up two pieces and weighted. The pieces were placed into ultrapure water at room temperature. After regular time (1, 3, 5, 10, 15, 20, 30, 45, 60, 150 and 180 min) the pieces were removed, get rid of surplus water on the surface and re-weighed.

The swelling ratio of the scaffolds was defined as the ratio of wet sample (w) to the initial weight of dry sample (w_0), see (E 1). Each value was averaged from four parallel measurements.

Water content was calculated as the ratio of weight increase ($w–w_0$) to the initial weight (w_0), see (E 2).

$$Swelling\ Ratio = \frac{w}{w_0}, [-]$$

(E 1)

$$Water\ Content = \frac{w - w_0}{w_0} \cdot 100, [\%]$$

(E 2)

3.5.3 Degradation

After swelling measurement the samples were put into vials containing ultrapure water. The hydrolytic degradation was simulated in incubator at 37 °C. After the regular time the samples were removed, get rid of surplus water on the surface and re-weighed every day for one week and then after larger interval of time. The degradation solution was changed for fresh one every measurement.

The degradation was calculated according to equation (E 3) where the W_D is the weight of swelled scaffold in the appropriate day and the W is the stabilized weight at the appropriete time during swelling. For degradation graphs was used the weight of the scaffold after 60 minutes of swelling at first day of the measurement.

Degradation graphs were created using analysis program Excel.

$$Degradation\ [\%] = 100 - \left(\frac{W_D \cdot 100}{W} \right) \qquad (E\ 3)$$

3.5.4 Fourier Transformed-Infrared Spectroscopy

FT-IR analysis of scaffolds and pure substances was conducted for the qualitative determination of bonds presented in collagen samples. Samples were measured in the form of lyophylized dry sponges. FT-IR spectra were obtained by Tensor 27 FT-IR Spectrum with an average of 64 scans in a spectral range of 4000–400 cm^{-1} using software Opus 6.5.

3.5.5 Differential Scanning Calorimetry

The thermal stability and kinetic behavior of the scaffoldss was studied using a TA Instruments differential scanning calorimeter – DSC Q2000. Thermal profile of samples was measured from 10 to 100 °C. Test data were analyzed with Universal Analysis software. Temperature and enthalpy calibration were performed by using high purity indium standard.

A small piece of dry collagen sponge (1 mg) was placed in DSC hermetic pan and a defined quantity of ultrapure water was added, so that the concentration in the sample was 95 %. Specimens were then hermetically sealed inside aluminum pans to minimize moisture loss during the DSC scan. All tests were run at a heating rate scan of 2 °C/min. An empty capsule was used as reference. During the DSC scan, the samples were first cooled from room temperature to 10 °C followed by ramping up to 100 °C. Transition temperature was determined as the onset value of the occurring endothermic peak. The value of denaturation enthalpy was calculated with respect to the mass of vacuum dried collagen matrices.

3.5.6 Biological Properties

In-vitro tests were performed at the Institute of Experimental Medicine, Prague. 30,000 cells (3T3 mouse fibroblasts) per sample were seeded on scaffolds for the 14 day test biocompatibility samples. Fibroblasts tend to fill spaces within tissues and form extracellular matrix.

8 kinds of samples were tested:

1. Coll – crosslinked pure collagen
2. Coll/PRP – crosslinked collagen with addition of platelet lysate (platelet rich plasma)

3. Compl – crosslinked collagen with a complex of chitin/chitosan-glucan (1: 1)

4. Compl/PRP – crosslinked collagen with a complex of chitin/chitosan-glucan + platelet lysate

5. CaOC – crosslinked collagen with oxidized cellulose (1: 1)

6. CaOC/PRP – crosslinked collagen with oxidized cellulose + platelet lysate

7. Chit – crosslinked collagen with chitosan (1: 1)

8. Chit/PRP – crosslinked collagen with chitosan (1: 1) + platelet lysate

Cell adhesion, proliferation and viability were tested during 14 days.

Carrier fitted fibroblasts were observed using confocal microscopy (CLSM) in the 1st, 7th and 14th day. Cells were fixed with frozen methanol and stained with fluorescent. Cell membranes were stained with $DiOC_6$ (Green λ_{ex} = 484 nm, λ_{em} = 501 nm), cell nuclei with propidium iodide (red λ_{ex} = 536 nm, λ_{em} = 623 nm).

Cell viability was determined by MTS assay. The incubation period with the reagent was 3 hours. Media absorbance was measured at 490 nm.

To determine the extent of proliferation of melanocytes, the PicoGreen method (dsDNA fluorescent dye) was chosen. Fluorescence was measured (λ_{ex} = 485 nm, λ_{em} = 528 nm).

4 RESULT AND DISCUSSION

4.1.1 Morphology and Porosity

Porous structures allow for optimal interaction of the scaffold with cells. Specifically, pore size determines the efficiency at which cells seed into the scaffold; small pores prevent the cells from penetrating the scaffold, whilst large pores prevent cell attachment due to a reduced area and, therefore, available ligand density. A scaffold with an open and interconnected pore network and a high degree of porosity (> 90 %) is ideal for the scaffold to interact and integrate with the host tissue [315].

The freezing proces during lyophilization initially produced the nuclei of ice crystallization and the nuclei gradually increased to final size of ice crystals. The frozen collagen material is dried with sublimating ice crystals under vacuum at a temperature below the ice freezing temperature. The differences of pore size and porosity of different collagen scaffolds demonstrate the change in hydrophilicity of the matrices and heat transfer rates which enabling homogenous crystallization of water. This property can be regulated by addition of different additive to collagen and by cross-linking prepared scaffolds.

The different morphology of the pure collagen sponges and with PRP is shown in Figure 14. The viscous solution of PRP was poured onto the sponge and relyophilized again. A homogenous network was formed by interaction of PRP particles with a matrix and the structure of the final scaffold was changed. Figure 15 represents clearly noticeable inclusion of the PRP particles into the structure and its wrapping by collagen substrate.

The morphology of all prepared scaffolds is shown in Appendix 1.

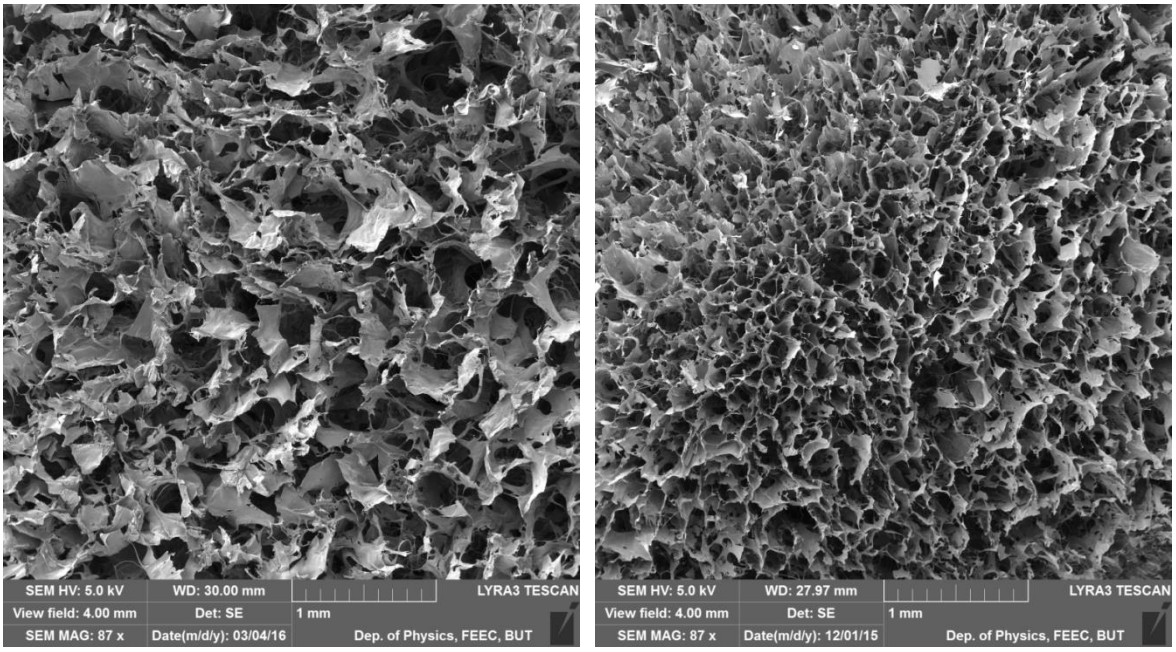


Figure 14: SEM image of collagen scaffold without PRP (left) and with PRP (right).

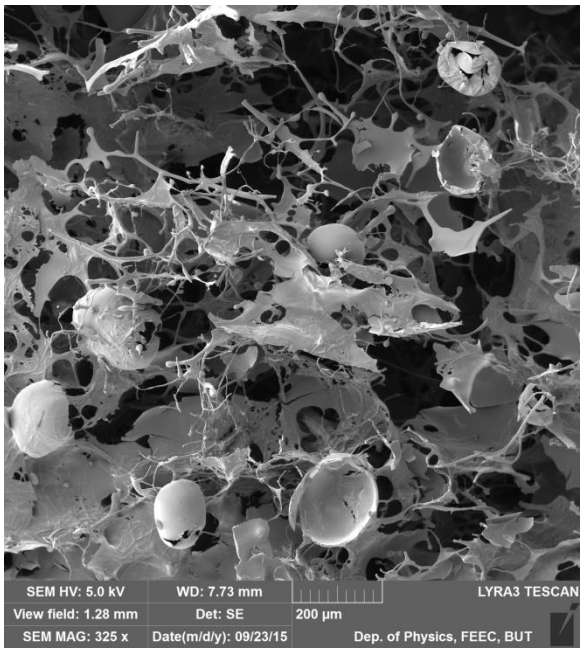


Figure 15: SEM image representing the structure of collagen/complex sponge enriched with PRP.

Pore size and porosity were observed in two sections of each scaffold, cross and longitudinal. The pore size distribution of all SEM images is shown in Appendix 2 and Appendix 3. From averages of pore size values of scaffolds was constructed graph in Figure 16. Error bars here show a range of pore sizes.

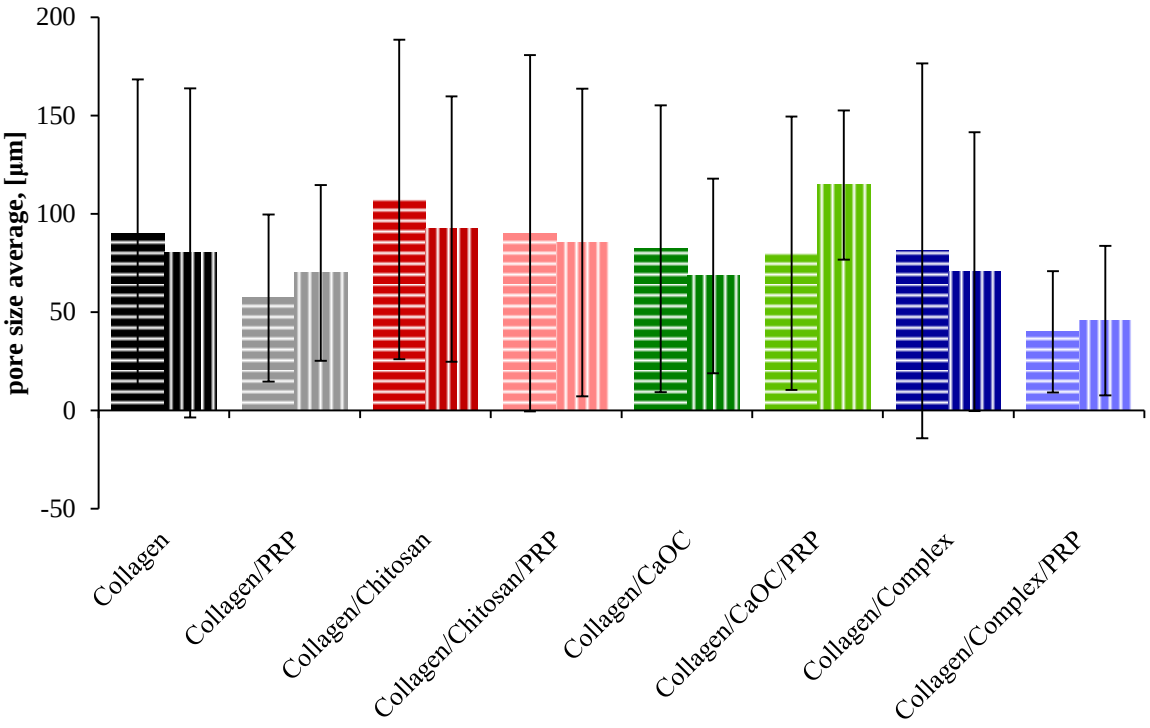


Figure 16: Pore size average for cross (left column) and longitudinal (right column) section.

Most human cells fall within a size range of 2–120 microns [316]. According to the Figure 16 average pore size of each prepared scaffold moves in this optimal range.

The smallest pores were formed in scaffold Collagen/Complex/PRP. In contrast, porosity value for this scaffold moved higher than most other scaffolds, as can be seen in Table 6.

Comparing the values presented in Table 6, scaffold with PRP had smaler pore size average values than coresponding scaffold without PRP, except the Collagen/CaOC scaffold. On the other hand, porosity increases with the addition of PRP in all cases. Collagen/CaOC/PRP scaffold could make an exception because of its fragility and partial demage to the surface structure of the scaffold while cutting, so the SEM image was not suitable for image analysis. By degradation evaluating it is the most unstable scaffold, as could be seen below in degradation results. The calculated pore size for this scaffold is thus not exact.

The collagen/aditive scaffolds had higher porosity in the comparison with pure collagen, which decreased after cross-linking in followed order: Collagen/CaOC > Collagen/Chitosan > Collagen/Complex corresponding to porosity of 65.2 % > 59.2 % > 57.5 %, respectively (Table 6). The porosity of samples increases for each with addition PRP.

Table 6: Total pore size average for both section (crosswise and longitudinal) and porosity of collagen scaffolds.

Sample	Pore size average, [μm]	Porosity, [%]
Collagen	85.05 ± 81.05	56.67 ± 0.94
Collagen/PRP	63.50 ± 43.60	60.65 ± 1.07
Collagen/Chitosan	99.75 ± 74.37	59.24 ± 2.94
Collagen/Chitosan/PRP	87.73 ± 84.44	59.62 ± 0.60
Collagen/CaOC	75.27 ± 61.21	65.22 ± 1.10
Collagen/CaOC/PRP	97.27 ± 53.76	66.19 ± 4.79
Collagen/Complex	75.88 ± 83.12	57.49 ± 3.23
Collagen/Complex/PRP	42.79 ± 34.47	63.44 ± 0.23

There is the hypothesis that the carboxylic groups of collagen ionically interacted with amino groups of chitosan, chitin-chitosan-glucan complex and especially PRP proteins. The hydroxyl groups of these compounds would be than sterically available to interact with water. This hydrophilic character of created sponges had thus better porosity than pure collagen.

According to the Figure 16 it is considered, that most samples do not have homogenous structure in whole volume, because the pore size averages vary for cross and longitudinal section. Transversal and longitudinal sections exhibit different locations in the volume of scaffold, which differ in structure, morphology and pore size distribution (Appendix 1, Appendix 2, Appendix 3).

4.1.2 Swelling Ratio and Water Content

Swelling properties of collagen scaffolds were studied in ultrapure water. In few minutes scaffold quickly absorbed maximum of water. After 60 minutes, the swelling ratios were constant in most cases.

Concerning the Figure 17, swelling ratios of collagen sponges enriched with PRP distincly decreased in comparison with collagen sponges without PRP. Here it shows that PRP make more compact structure and scaffold could not soak inside much water because of absorbation of PRP firstly.

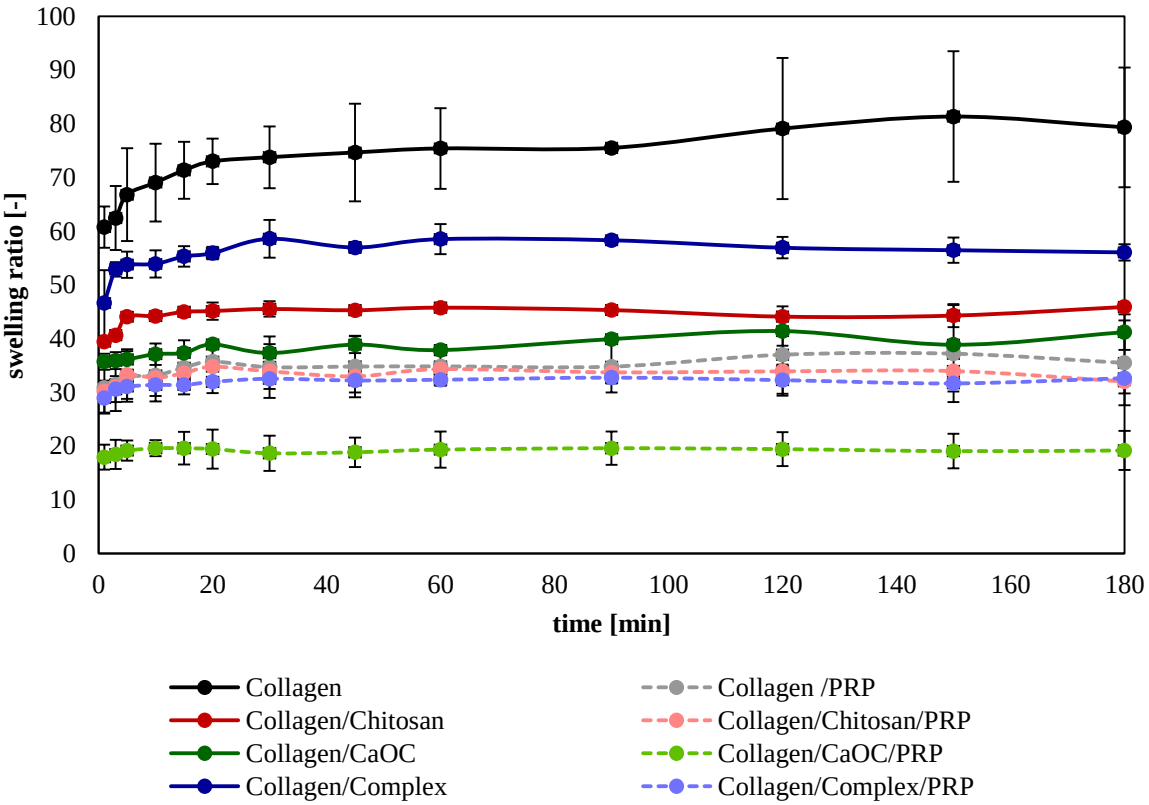


Figure 17: Swelling ratio curves of collagen based sponges in water from the second serie (all crosslinked).

The water content was studied as amounts of water absorbed by swelling. The highest amounts of water absorbed pure collagen scaffold (98.67 %) in comparison with collagen sponges with additives (Figure 18). The lowest value of water absorbed Collagen/CaOC/PRP sponge. The dependent of water content on time were very similar to these of swelling ratio curves and thus they are shown in Appendix 4.

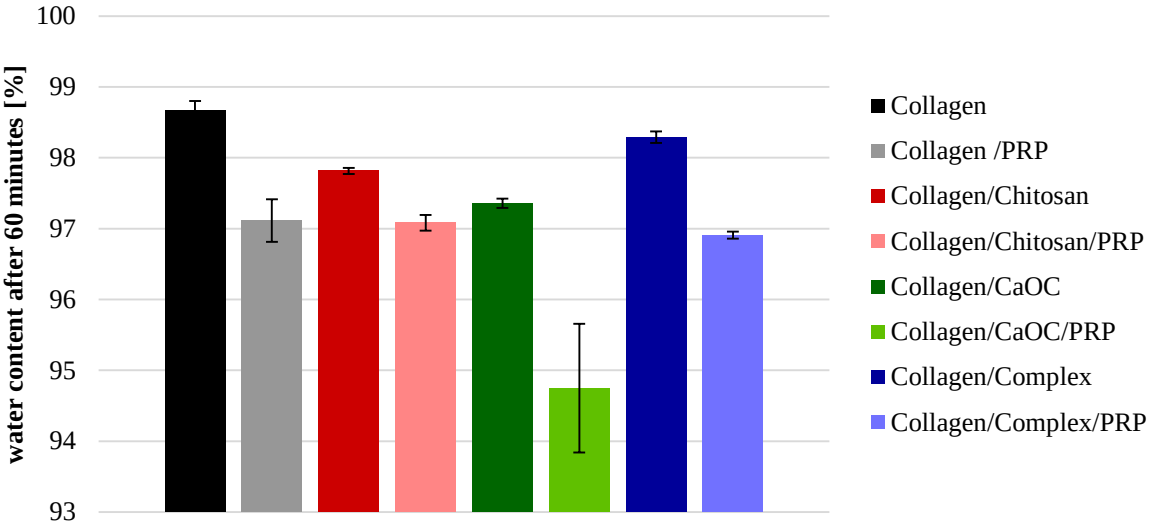


Figure 18: Water content of collagen based sponges in water after 60 minutes.

The swelling properties of collagen sponges modified with bioactive aditives decreased in followed order Collagen > Collagen/Complex > Collagen/Chitosan > Collagen/CaOC.

Water content of Collagen/PRP, Collagen/Chitosan/PRP and Collagen/Complex/PRP modified scaffolds move at very similar values. The exception here is Collagen/CaOC/PRP which absorbed the least amount of water. Large error bars at Collagen/CaOC/PRP scaffold are caused by changing the structure during the swelling where the scaffold did not keep shape and was too soft like a gel.

Using the swelling method the influence of crosslinking at the scaffolds with additives CaOC and complex was also studied. According to Figure 19, there is marked decline of swelling ratios at non-crosslinked samples of tens. Non-crosslinked samples are not able under the action of water to keep the shape.

The most unstable was non-crosslinked scaffold modified with CaOC and PRP, because it degraded during the swelling in 60 minutes, which can be seen in the Figure 19.

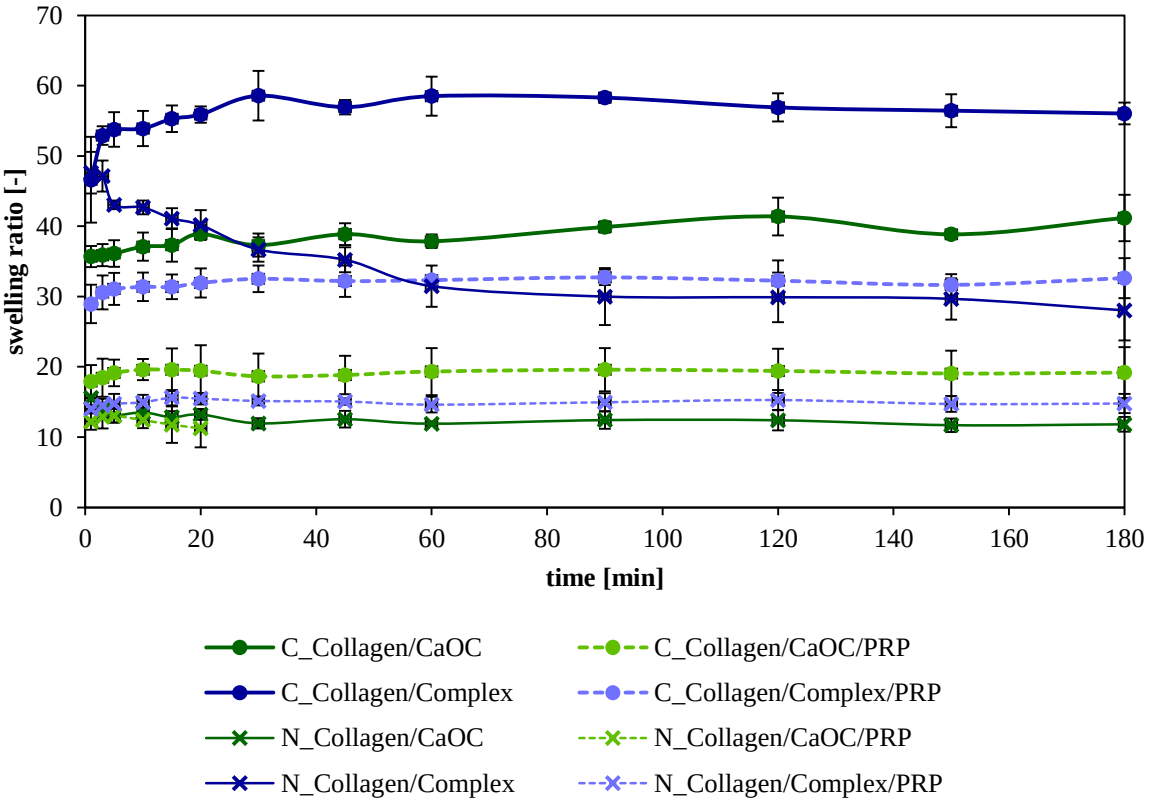


Figure 19: Swelling ratio curves of crosslinked (C) and non-crosslinked (N) collagen based sponges with aditives CaOC and complex.

It may seem, that the scaffolds having higher porosity absorb higher volume of water and resulting in a bigger number of swelling ratios. If the swelling properies depended only on the free volume in pores, porosity values for scaffolds would be in the same order. But according to determined values in Figure 20, the trend of swelling ratio is exactly opposite to the porosity trend (except Collagen/CaOC) of scaffold. The greater the porosity, the less mass in the scaffold. This means that water is not retained in the empty areas of pores, but interacts with the scaffold and stays absorbed therein.

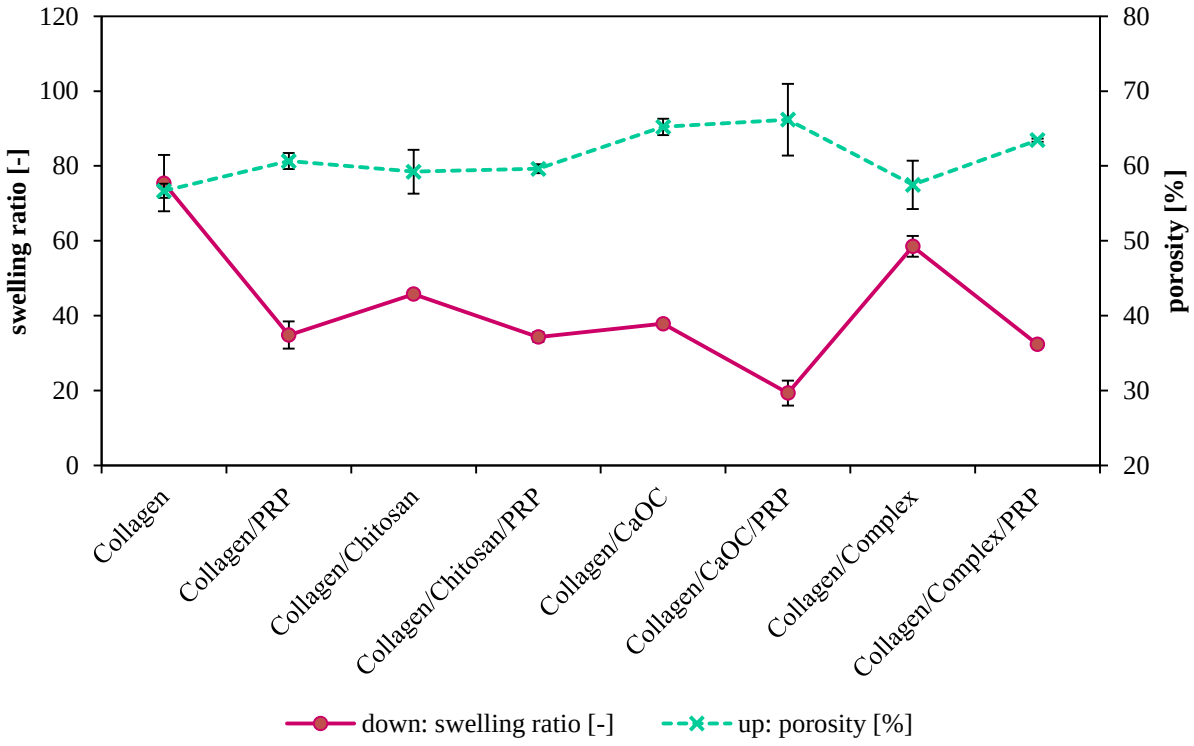


Figure 20: Trend of swelling ratio in water after 60 minutes (down) and porosity (up) of collagen based sponges prepared in the second serie (all crosslinked).

4.1.3 Degradation

The degradation of collagen scaffolds was simulated in incubator in ultrapure water at 37° C. Degradation properties were studied on the first serie of prepared samples, where scaffolds with additives complex and CaOC were not crosslinked.

Figure 21 and Figure 22 represent the dependences of weight loss on time for unmodified and modified samples with PRP, respectively, which were washed out with clean water during the degradation evaluation.

By stability test the effect of the crosslinking agent was confirmed, when non-crosslinked samples disintegrated first. Hydrolytic stability of non-crosslinked samples after a certain time increased in order Collagen/CaOC/PRP < Collagen/Complex/PRP < Collagen/CaOC < Collagen/Complex. The highest resistance of collagen sponges to hydrolytic degradation in water exhibited crosslinked samples and was not disintegrated after a period of 300 days.

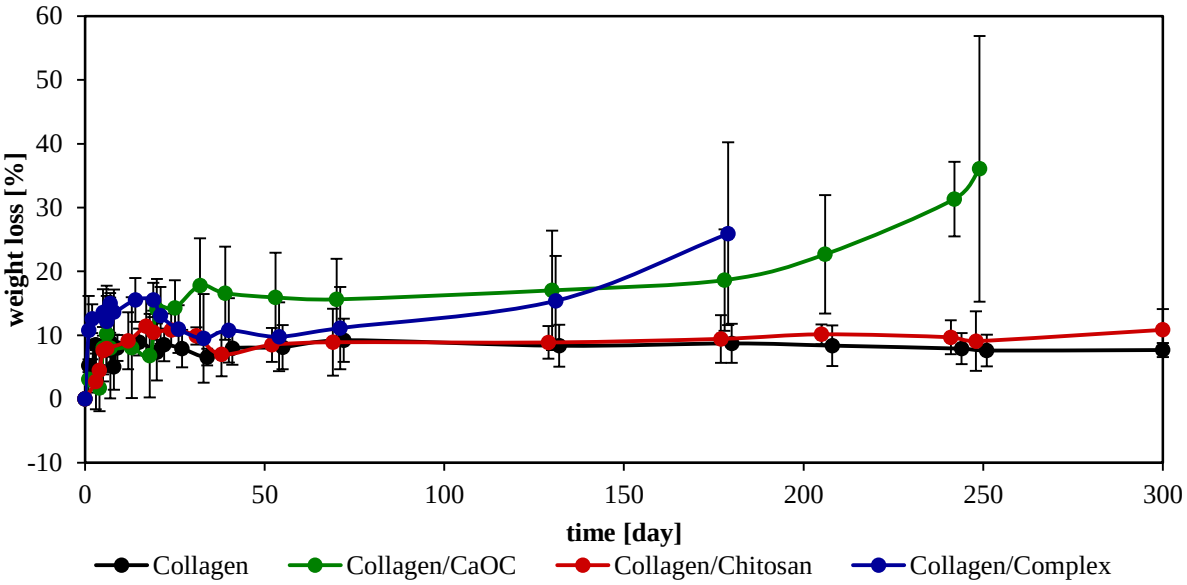


Figure 21: The dependence of the weight loss on the degradation time for modified collagen sponges without PRP.

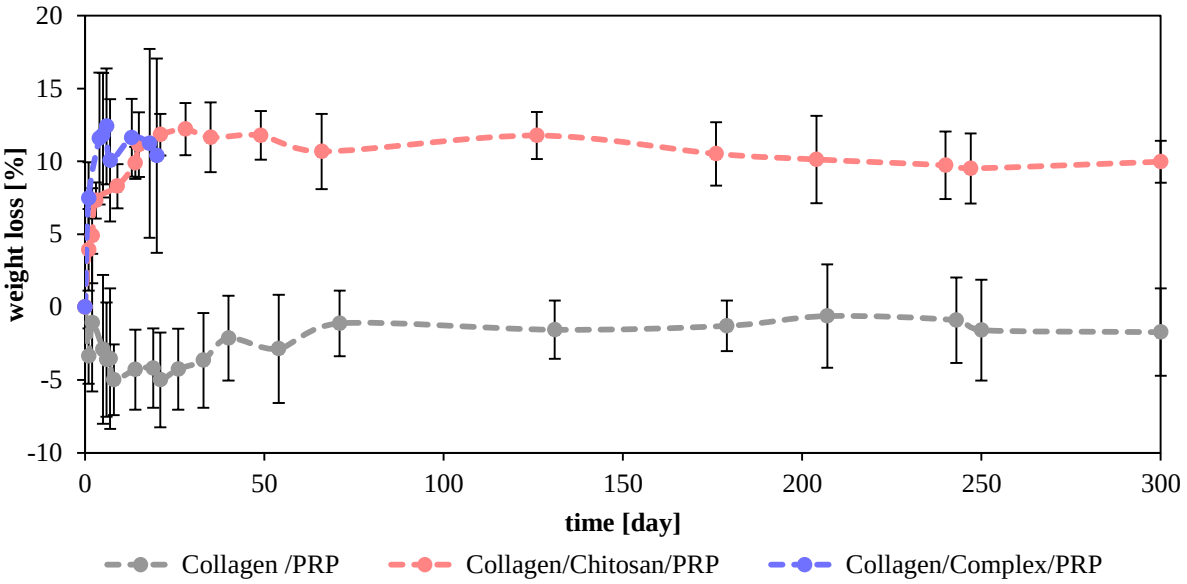


Figure 22: The dependence of the weight loss on the degradation time for modified collagen sponges with PRP.

The sample Collagen/PRP has seemed to bee still swelling after first day, because it had minus weight loss values.

The highest degradation rate was recorded at Collagen/CaOC/PRP sponges which were fully degraded within 1 hour in water. Full degradation in 26 days belong to sample Collagen/Complex/PRP. It seems like addition of PRP makes the structure of scaffold more unstable, although swelling abbility decrease with addition of PRP and samples thus do not fit in the volume of water as scaffolds without PRP.

PRP particles are soluble in water and after their washing out of scaffold pores, there is an empty space, where water can penetrate. The degradation then proceeds not only

on the surface but also throughout the entire volume and the disruption of sponge accelerates. Samples which were not enriched with PRP exhibited much higher stability in time.

4.1.4 Fourier Transformed-Infrared Spectroscopy

The expected types of bond linkages presented in the samples were confirmed using infrared analysis. Figure 23 shows the infrared spectrum of pure collagen mass.

Pure collagen displays bands at 3 305, 1 631, 1 544 and 1 237 cm^{-1}, which are related to the amide A, I, II and III bands of collagen, respectively, and which are characteristic for the peptide bond. The amide A is merged with the NH_2 stretching. The amide I absorption arises predominantly from protein amide C=O stretching vibrations, the amide II absorption is made up of amide N–H bending vibrations and C–N stretching vibrations; the amide III peak is associated to CH_2 residual groups from glycine and proline and it is complex, consisting of components from C–N stretching and N–H in plane bending from amide linkages [317].

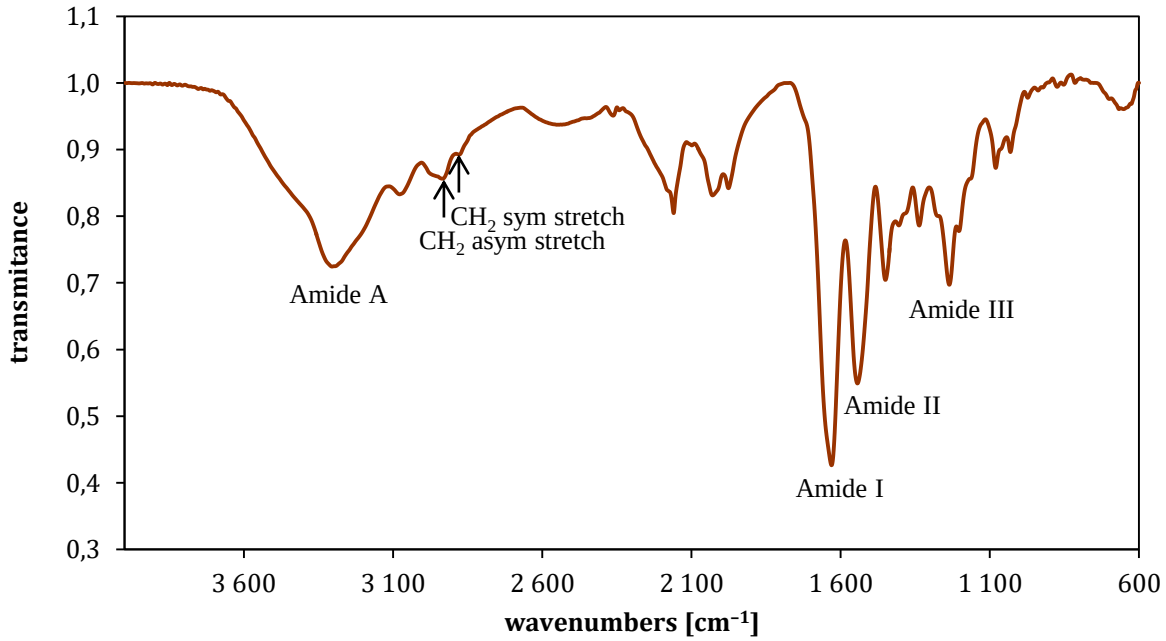


Figure 23: IR spectrum of pure collagen.

As for pure collagen, samples in the form of sponges prepared by lyophilization, characteristic bands were interpreted. Band of amide I was found to be located at 1 633 cm^{-1}, amide II band is in region of wavenumber at 1 547 cm^{-1} and band of amide III is located in wavenumber of 1 239 cm^{-1} (Figure 24).

In the collagen scaffold spectra, there is a difference that a gentle double amide I peak was observed between 1 633 cm^{-1} and 1655 cm^{-1} (Figure 24). This band is linked to the extent of intermolecular interactions in collagen and collagen-like peptides: triple helix (1660 cm^{-1}), C=O imino type group (1637 cm^{-1}), and COOH (1685 cm^{-1}) [318].

Area and location of individual band maximum is changed according to the changes in the structure of collagen.

Porous structure formation which increases intermolecular interactions in collagen, is associated with broadening and slight shift to lower wavenumber of the amide A, decrease in intensity and slight shift to higher wavenumber of amide III peak and band broadening and shift of amide I peak to higher wavenumber. All these bands reflect the secondary structure of the sample.

Denaturation of collagen, on the other hand, is manifested by reduction in intensity of band of amide A, amide I, amide II and amide III and decrease of amide I components in wavenumber of 1630 cm^{-1} and 1660 cm^{-1} [319] and this does not corespond with the data obtained. The lyophilization process changes the secondary structure only marginally.

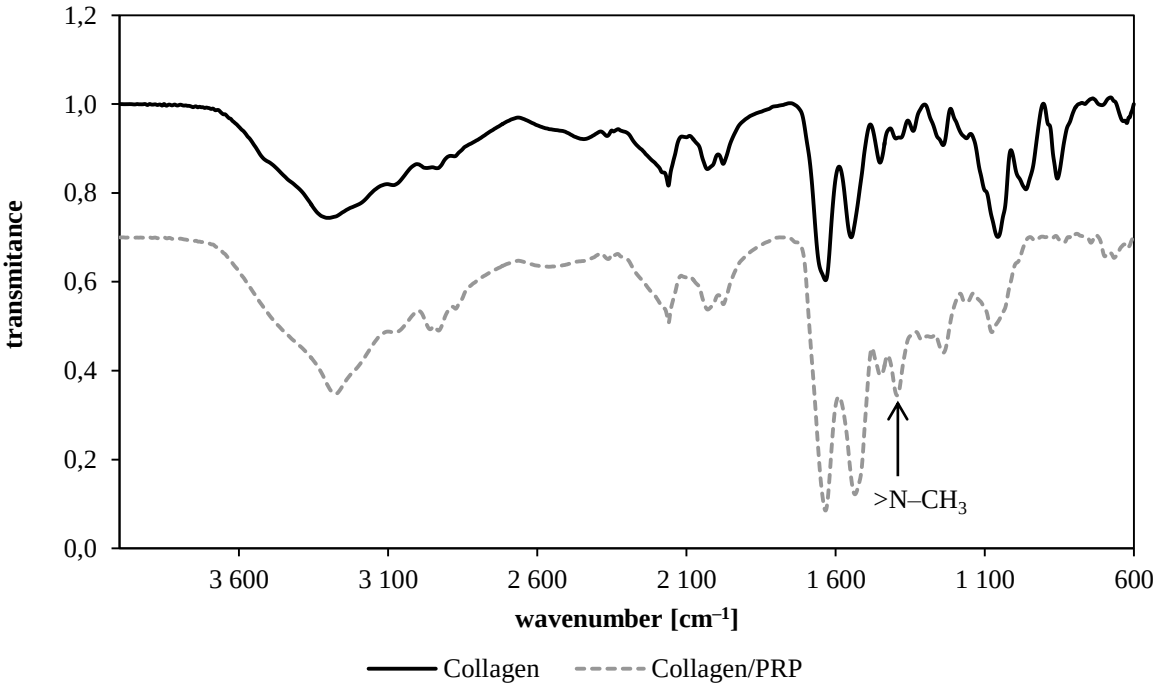


Figure 24: IR spectra of collagen and collagen/PRP sponge.

Characteristic bands of oxycellulose – Ca can be assigned to bands in the region 1 593 cm^{-1} and 1 732 cm^{-1} (Figure 25, Table 7). The presence of a strong absorption band at 1 593 cm^{-1} confirms the presence of COO^- group. The band of wavenumber 1 732 cm^{-1} corresponds to the vibration of carboxyl group COOH, this functional group with oxidized cellulose is bonded to carbon C6 in the pyranose ring.

IR spectrum of oxidized cellulose also showed a broad absorption band around 3 294 cm^{-1} that confirms the stretching frequency of the –OH group and associates to the presence of water in the sample. The bands around 1 416 and 1 282 cm^{-1} are assigned to C–H deformation vibration from secondary alcohol and –OH bending vibration, respectively. The band at 1 155 cm^{-1} is due to antisymetric C–O bridge stretching and band at 1 023 cm^{-1} belongs to CH_2–O–CH_2 stretching, both in the pyranose ring [320].

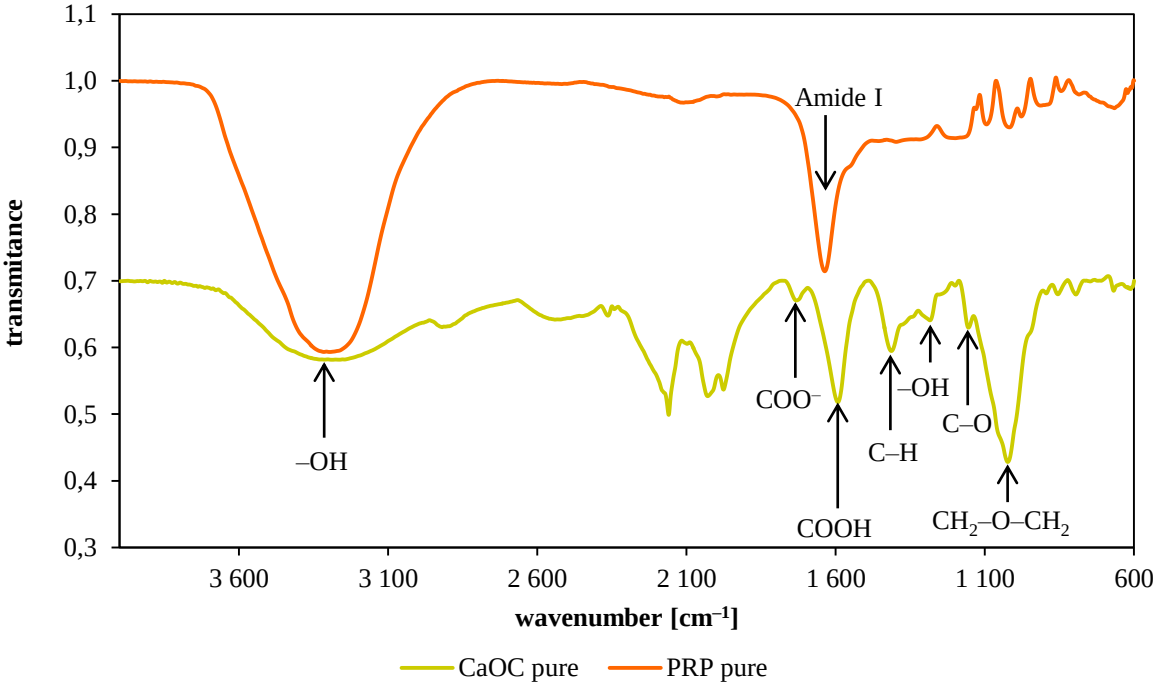


Figure 25: IR spectrum of pure calcium oxidized cellulose and pure PRP.

Table 7: Summarization of some characteristic bands and their wavenumbers [cm^{-1}] for measured samples with calcium oxidized cellulose.

Substance	**–OH**	**COOH**	**COO^-**	**–CH def**	**C–O stretch**	**CH_2–O–CH_2**
CaOC pure	3 247, 1 284	1 732	1 593	1 413	1 155	1 023
Collagen/CaOC	3 303, 1 284	1 724	1 598	1 416	1 156	1 030
Collagen/CaOC/PRP	3 275, 1 283	–	–	–	1 155	1 033

When compared infrared spectra of collagen sponge and collagen after the modification with CaOC significant changes are evident (Figure 26).

Free amino groups present in collagenous sample may react with a suitable reagent to form compounds containing functional groups. Here in the collagen/CaOC scaffold, CaOC was as the reagent. CaOC reacts with amino group of collagen forming peptide bond (Figure 26). CaOC contains two functional groups absorbing in the infrared region, both COO^- (1 593 cm^{-1}) and COOH (1732 cm^{-1}). These functional groups are not involved in collagen samples without CaOC.

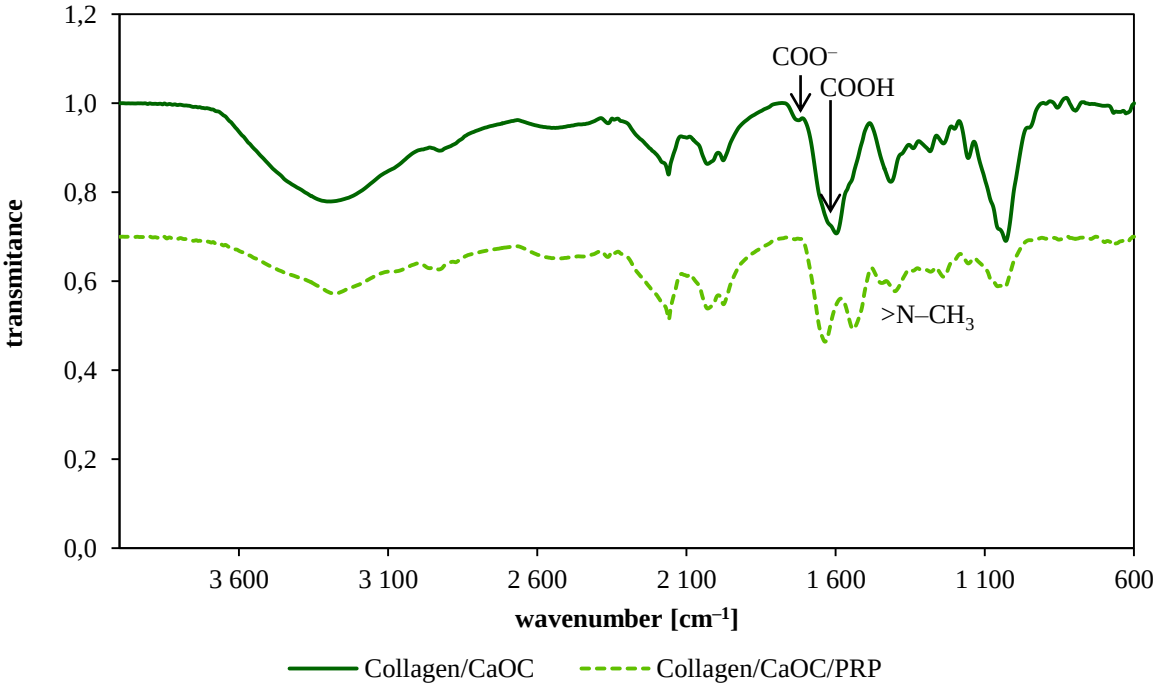


Figure 26: IR spectra of collagen, collagen/CaOC ang collagen/CaOC/PRP sponges.

The spectrum of chitosan shows some typical bands around 3 354, 1 654, 1 590 and 1 026 cm^{-1} which correspond to amides A, I, II and CH_2–O–CH_2 stretching in the pyranose ring, respectively (Figure 27). The amide I arises from C=O stretching; the amide II arises from –NH torsion groups. The shoulder at 1 654 cm^{-1} suggests that chitosan comes from a partial deacetylation process [317], [321].

At wavenumbers between 1 421 and 1 061 cm^{-1} absorbed groups characteristic for alcohols. Bands at 1 421 and 1 375 cm^{-1} belong to C–H deformation vibration from bounded and free secondary alcohols, respectively. C–O stretching from tertiary alcohol (bridge in the pyranose ring) showes band at 1 150 cm^{-1} and C–O stretching from primary alcohol at 1 061 cm^{-1}.

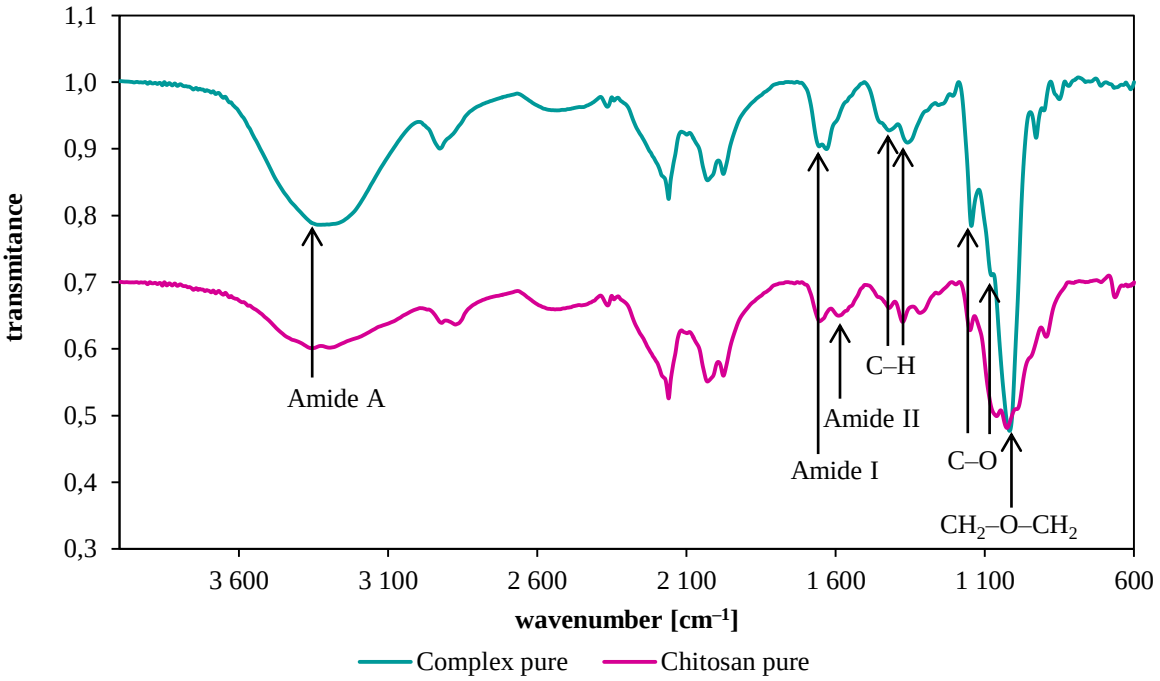


Figure 27: IR spectrum of pure complex and pure chitosan.

According to Figure 27 and Table 8 it is obvious, that pure chitosan and pure complex absorbed at wavenumbers, which are characteristic for the same vibrations. Pure complex sprectrum lack the only band coresponding to amide II. As discussed above, the amide II arises from –NH torsion groups. Complex is copolymer created from chitin, chitosan and glucan. Molecule of glucan does not contain any amines and amides and its structure could affect spetctrum of pure complex, where the intensity of amide II band distincly decreased.

Table 8: Summarization of some characteristic bands and their wavenumbers [cm^{-1}] for measured samples with chitosan and complex.

Substance	**Amide A**	**Amide I**	**Amide II**	**C–H def**		**C–O stretch**		**CH_2–O–CH_2**
Chitosan	3 354	1 654	1 590	1 421	1 375	1 150	1 061	1 026
Collagen/Chitosan	3 293	1 632	1 550	1 398	1 338	1 158	1 062	–
Collagen/Chitosan/PRP	3 278	1 635	1 538	–	–	1 159	1 076	1 033
Complex pure	3 325	1 631	–	1 420	1 359	1 145	1 077	1 018
Collagen/Complex	3 296	1 633	1 548	–	1 339	1 147	1 079	1 024
Collagen/Complex/PRP	3 277	1 635	1 540	–	–	1 148	1 077	1 031

The FT-IR spectra of the collagen/chitosan sponges are shown in Figure 28. The behavior of the bands located around 1 635–1 450 cm^{-1}, which corresponded to the collagen, indicated that there is an interaction between the polymers. This explains the compatibility between collagen and chitosan which results in good sponge homogenity. The same statement hold collagen/complex sponges as well.

The modification in observed bands amplitude suggests the presence of molecular interactions, mainly hydrogen bonding between the two polymers, also exhibiting miscibility and alternation of each of the components. Besides hydrogen bonding, physicochemical

characteristics of chitosan, molecular weight and deacetylation degree, can affect the conformational changes of biomolecules in the matrix of scaffold [322].

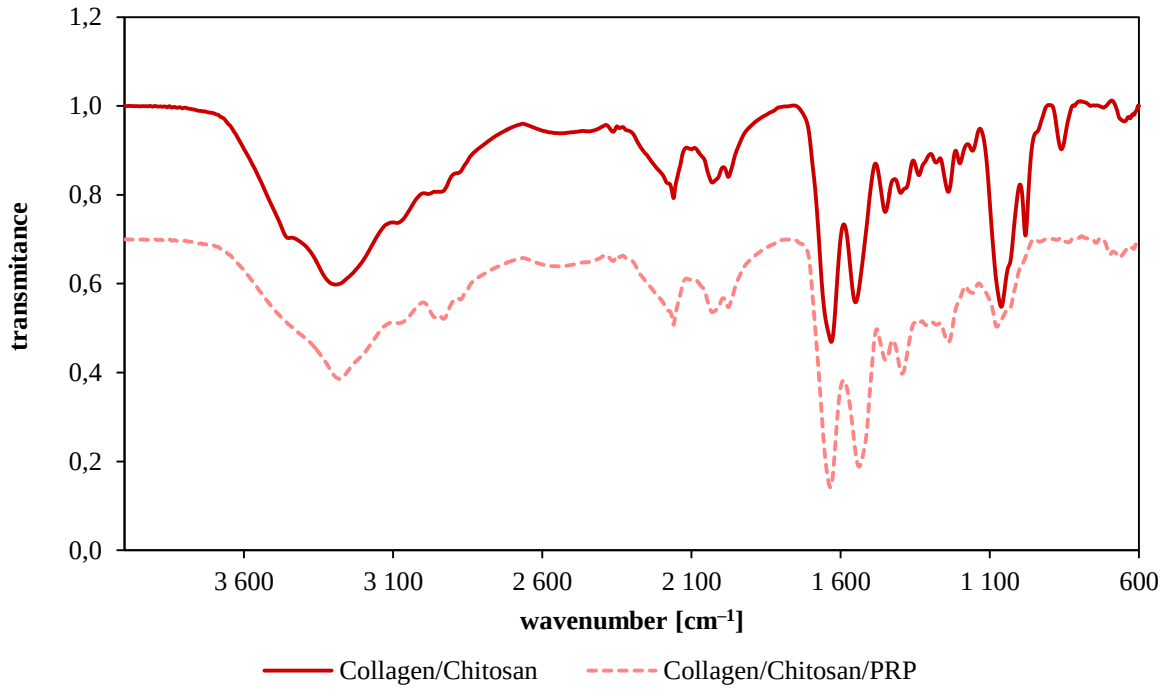


Figure 28: IR spectra of collagen/chitosan and collagen/chitosan/PRP sponge.

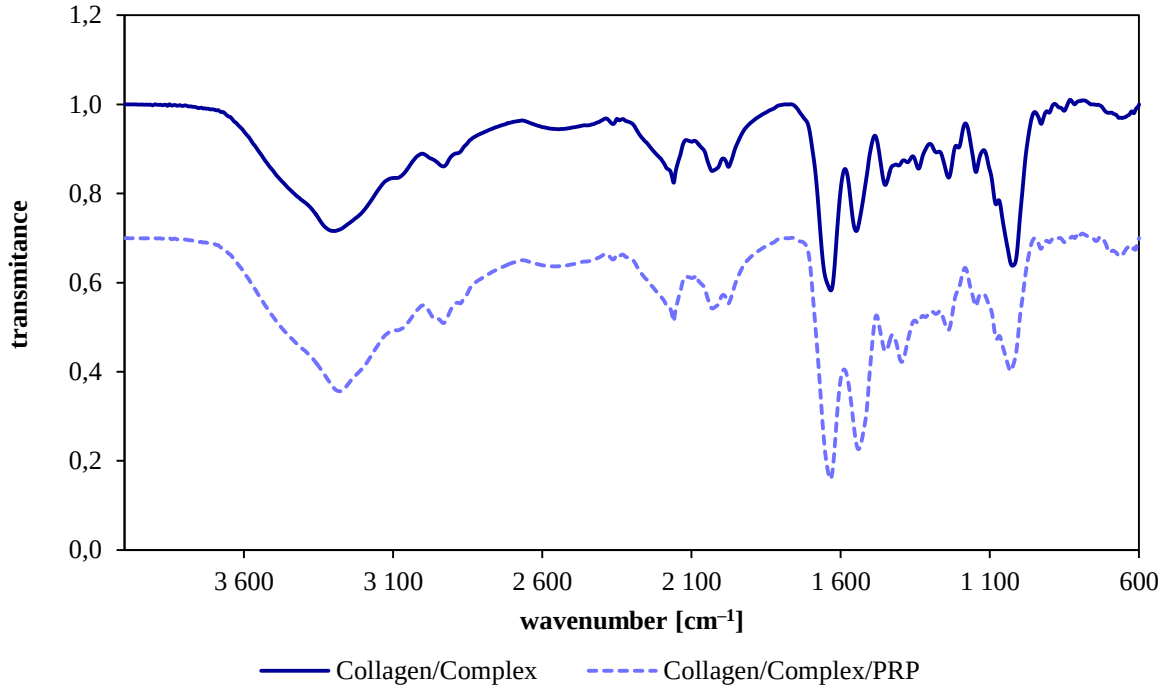


Figure 29: IR spectra of collagen/complex and collagen/complex/PRP sponge.

The frequencies at which major peak occurred for individual mixtures of collagen/additive and pure substances are summarized in Table 9.

Table 9: Summarization of characteristic bands and their wavenumbers[cm^{-1}] for measured samples.

		Amid A	**Amid I**	**Amid II**	**Amid III**	**$-CH_3$ asym**	**$>N-CH_3$**
Pure substance	**Collagen pure**	3 305	1 631	1 544	1 236	1 450	–
	PRP pure	3 316	1 637	–	–	–	–
Scaffold	**Collagen**	3 297	1 633	1 547	1 239	1 450	–
	Collagen/PRP	3 276	1 634	1 534	1 239	1 450	1 394
	Collagen/Chitosan	3 293	1 632	1 550	1 239	1 450	–
	Collagen/Chitosan/PRP	3 278	1 635	1 538	1 239	1 450	1 394
	Collagen/CaOC	3 303	–	1 552	1 239	–	–
	Collagen/CaOC/PRP	3 275	1 636	1 542	1 238	1 446	1 401
	Collagen/Complex	3 296	1 633	1 548	1 238	1 450	–
	Collagen/Complex/PRP	3 277	1 635	1 540	1 239	1 450	1 395

Comparing the spectra of unmodified and modified scaffolds according to the Figure 24, Figure 28 and Figure 29 it is obvious that the major bands of mixtures have the same resulting position with small deviations in the order of units of cm^{-1}.

IR spektra of all samples enriched with PRP assigned with only one characteristic band around 1 395 cm^{-1}. PRP is protein based and contains free groups which can interact with free groups of collagen. By reacting two substances of a protein character arises amine for which is typical band in this area and the vibration belongs to $>N-CH_3$, where symetric deformation vibration C–H absorbed.

Pure PRP spectrum showed a signal with several bands, where appeared only two intense bands at wave numbers 3 316 and 1 637 cm^{-1} that belong to stretching frequency of the –OH group and C=O stretching vibrations, respectively.

4.1.5 Differential Scanning Calorimetry

DSC measurement was performed on the first serie of prepared samples, where scaffolds with additives complex and CaOC were not crosslinked.

With DSC the helix-to-coil-transition of collagen fibers can be measured as a temperature dependent endothermic signal, which indicates the extent of intermolecular crosslinking [323]. The endothermic peak is due to the denaturation of the higher order (secondary and tertiary) structures in the pongess. A denaturation mechanism was proposed in which the native state partially uncoils via a reversible step, followed by an irreversible step during which bound water is released and local unfolding occurs, producing a random, uncoiled state in the fibrils [324]. The area underneath the denaturation curve represents the amount of energy required to denature the sponges.

The results indicated differences in the hydrothermal stability of collagen scaffolds depending on the type of additives and the degree of crosslinking.

Non-crosslinked collagen sponges (modified by CaOC and complex, Figure 31) exhibited a lower stability, when compared with the crosslinked samples, Figure 30. According to Table 10, for crosslinked Collagen/PRP sponge the highest transition temperature of 71.0 °C was detected. The addition of bioactive substance causes a decrease of transition temperature due to its hydrophilic nature.

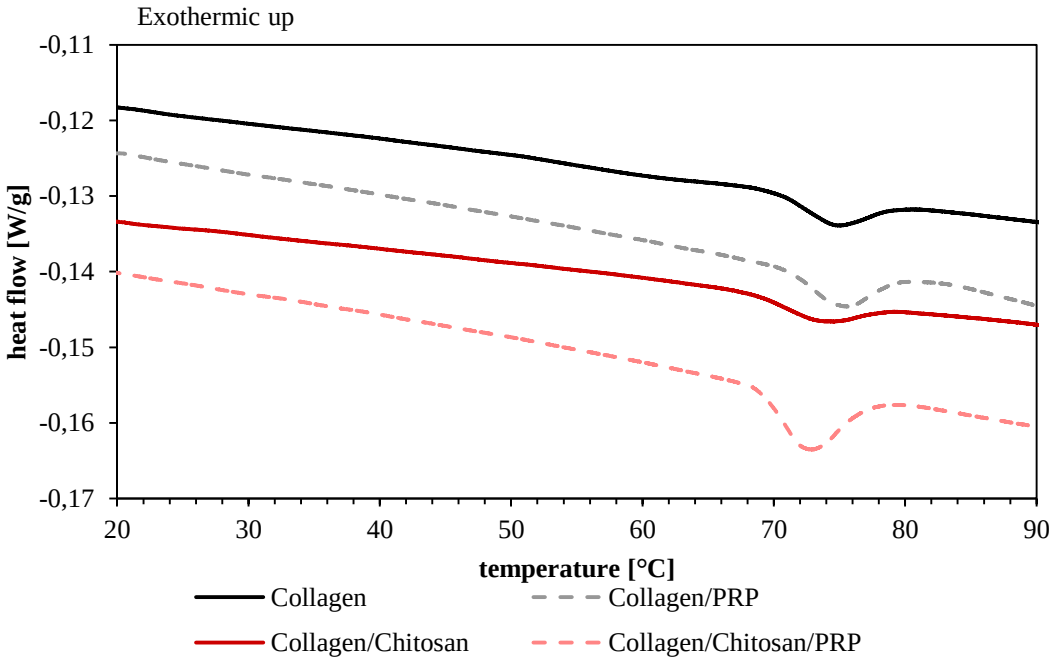


Figure 30: DSC traces for crosslinked collagen sponges.

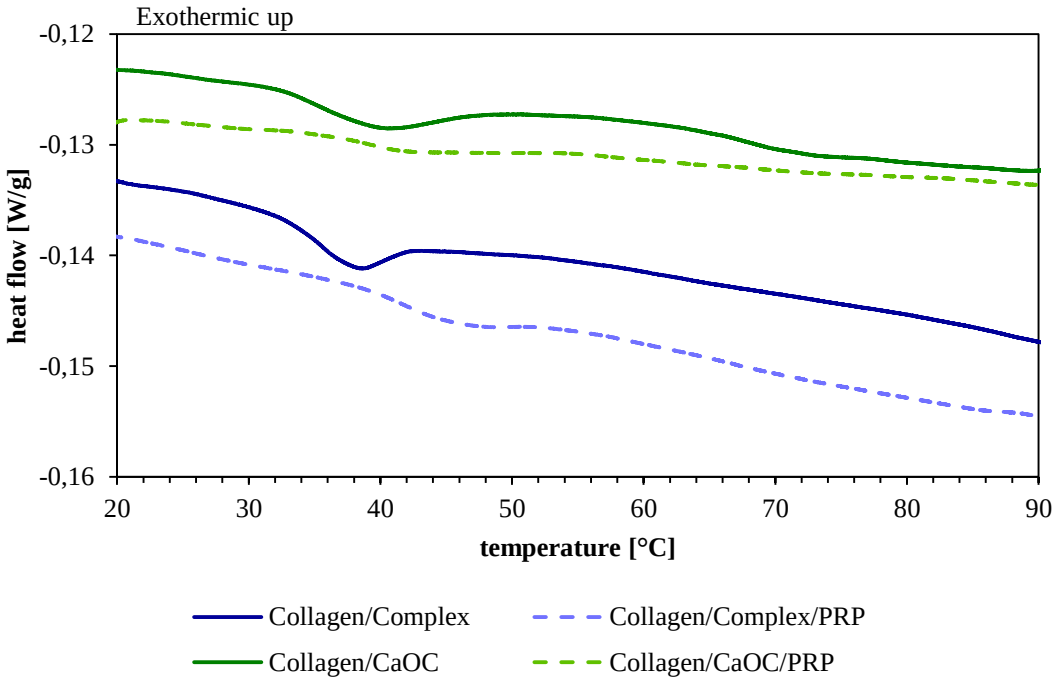


Figure 31: DSC traces for non-crosslinked collagen sponges.

Table 10: Onset and melting temperatures[°C] and enthalpies [J/g] of non-crosslinked and crosslinked collagen sponges modified with additives.

Sample		**t_onset [°C]**	**t_melting [°C]**	**ΔH [J/g]**	**w_dry mass [%]**
Non-crosslinked	Collagen	48.2	55.6	0.825	4.94
	Collagen/CaOC	32.2	40.0	0.819	4.71
	Collagen/CaOC/PRP	36.9	42.4	0.264	4.92
	Collagen/Complex	33.2	38.3	0.512	5.11
	Collagen/Complex/PRP	38.9	45.8	0.448	4.83
Crosslinked	Collagen	70.3	74.8	0.596	5.13
	Collagen/PRP	71.0	75.3	0.713	5.11
	Collagen/Chitosan	68.1	73.6	0.676	5.22
	Collagen/Chitosan/PRP	68.9	72.7	1.284	4.90

In all cases, sample enrichment with PRP increases the transition temperature. As shown in the SEM images, PRP particles are incorporated into the structure of the scaffold. PRP contains other proteins which are involved in thermodynamic processes during sample heating and changes transition temperature.

For non-crosslinked samples, transition enthalpies decreased with PRP enrichment. For the crosslinked samples, a higher transition enthalpy was found at samples enriched with PRP. Enthalpy depends on the amount of dry matter in the specimen. In the case of the uncrosslinked samples, after washing of the PRP of the sample, hydrolytic degradation in the whole volume proceeds much faster than in non-crosslinked samples. For this reason, the determined onset temperature can not be called the denaturation temperature, when non-crosslinked samples are taken into account.

4.1.6 Biological Properties

The viability of culture fibroblasts on scaffold depending on the use of additives was tested using the MTS assay on the 1st, 3rd, 7th, 10th and 14th day of experiment. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is reduced by living cells to a soluble formazan product. The reduction of MTT to formazan is limited in cytotoxic damage to or destruction of the cells. The degree absorbance is directly proportional to the number of live cells, i. e. the darker the color of the solution, the higher the percentage of live cells [325].

The best cell viability exhibited samples modified with bioactive additive and enriched with PRP, Collagen/Complex/PRP, Collagen/CaOC/PRP and Collagen/Chitosan/PRP (Figure 32).

Pure crosslinked collagen scaffold showed the viability of fibroblasts higher than the same scaffold enriched with PRP, but the cell viability on the 14th day significantly decreased (Figure 32). The decrease on the 14th day showed most samples apart from Collagen/Complex and Collagen/CaOC/PRP. The number of live cells was growing proportionally to the days for these two scaffolds.

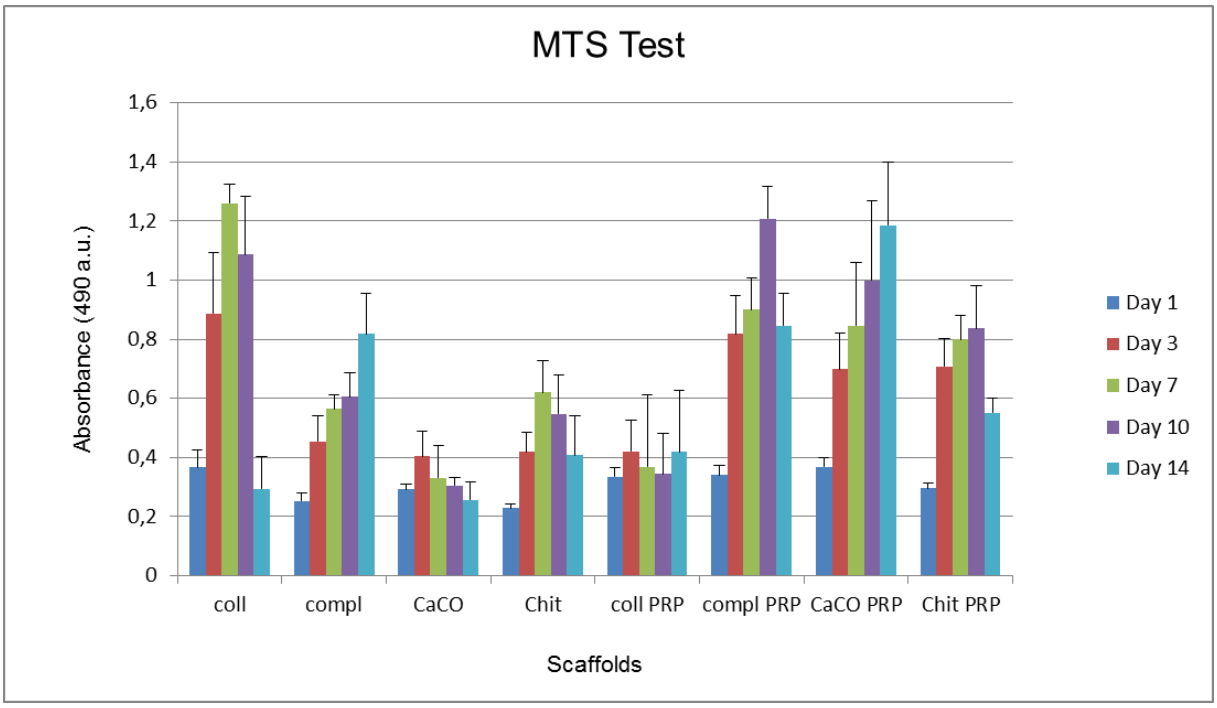


Figure 32: Cell viability by MTS test. Day 1, 3, 7, 10 and 14.

Influence of additive on the proliferation of fibroblasts was measured by PicoGreen method on the 1st, 3rd, 7th, 10th and 14th day of experiment which determines DNA content in samples. PicoGreen is a reagent for quantifying dsDNA which provides a stable measurement parameter. The measurement is based on fluorescence enhancement of the dye upon binding to dsDNA. The relative fluorescence units measured correlate with the number of cells present. Cell number and PicoGreen fluorescence exhibit a linear relationship for low and medium cell densities, but which is lost for high cell densities [326].

Scaffolds modified with CaOC and chitosan showed the best proliferation properties.

On the other hand, unmodified scaffolds and scaffold modified with complex exhibited distinctly lower amount of absorbed PicoGreen (Figure 33). According to Table 6, Collagen/PRP and Collagen/Complex/PRP had the smalest pore size. A change in surface topography can influence cell behavior substantially. Micron-scale roughness has been shown to modify cellular responses in cell culture and to modify biocompatibility and tissue attachment significantly [327]. This means that poliferation is influenced also by pore size of scaffold, because cells need to move through the structure.

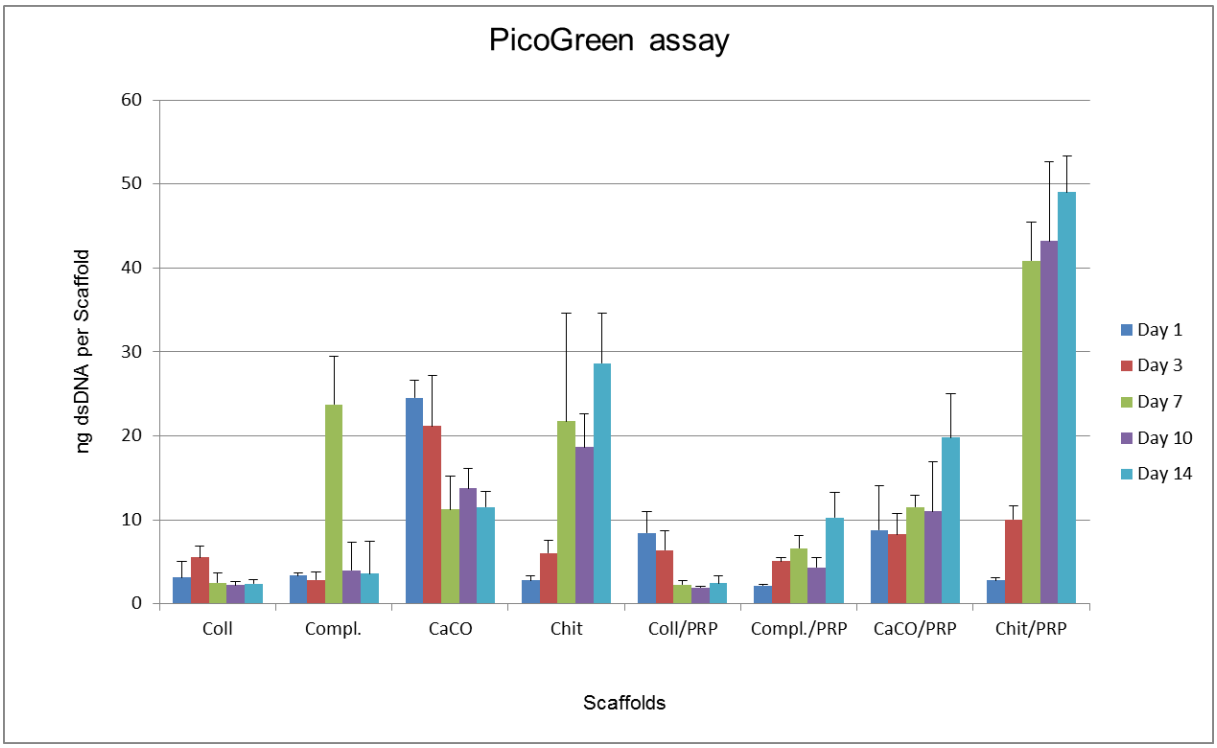


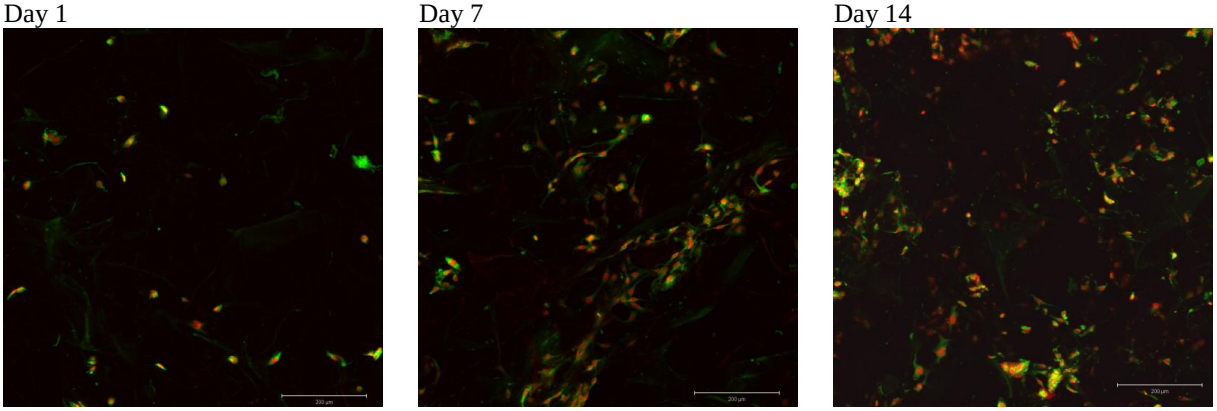
Figure 33: Cell proliferation by PicoGreen test. Day 1, 3, 7, 10 and 14.

Figure 34 presents the visualization of fibroblasts at the microporous Collagen/Chitosan and Collagen/Chitosan/PRP media by confocal laser scanning microscopy.

The visualizations of other scaffolds can be found in Appendix 5, Appendix 6 and Appendix 7.

A)

Day 1 Day 7 Day 14



B)

Day 1 Day 7 Day 14

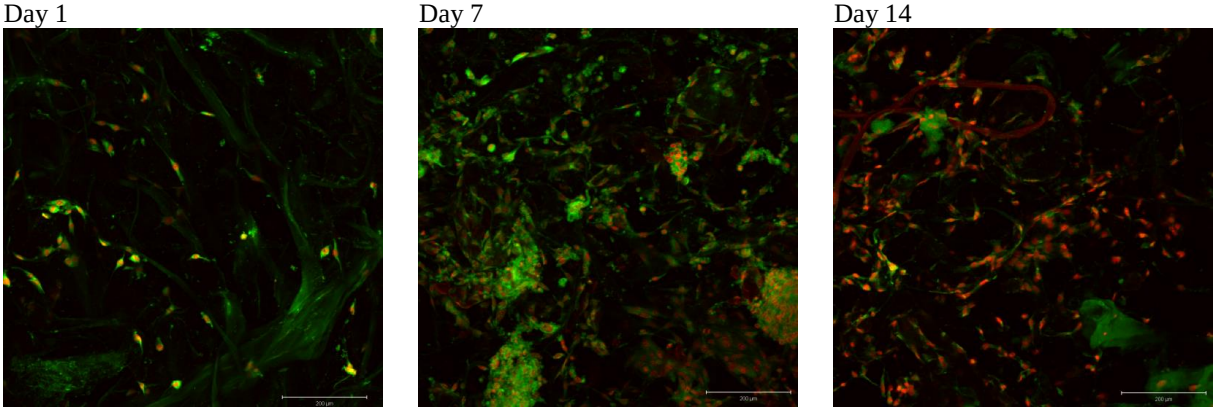


Figure 34: Adhesion and proliferation of the 3T3 fibroblasts on the A) Collagen/Chitosan and B) Collagen/Chitosan/PRP scaffold, $DiOC_6$/Propidium iodide staining ,200 micron scale.

5 CONCLUSION

In this work, the preparation of collagen scaffolds suitable for tissue engineering was investigated. The starting concentration of collagen equal to 1% was used. Consequently, the collagen solutions were modified with chitosan, calcium oxidized celulose and chitin/chitosan-glucan complex. Two series of samples with the collagen/additive weight concentration of 0.5 % were prepared. In the first serie, samples modified with calcium oxidized celulose and chitin/chitosan-glucan complex were not crosslinked. The samples of the second serie, all scaffolds were lyophilized followed by crosslinking process with EDC/NHS then again relyophilized. Each sample was prepared in the form without growth factors and in the form enriched with PRP. Enrichment was realized on lyophilized samples after crosslinking process if performed.

Concerning the characterization, morphology, pore size, porosity, swelling ratio, degradation time, qualitative determination and biological properties have been investigated.

The different morphology of the pure collagen sponges and with PRP was observed. A homogenous network was formed by interaction of PRP particles with sponge and the structure of the final scaffold was changed. The PRP particles incorporated into the structure and wrapped by collagen substrate.

An average pore size of each prepared scaffold pictures moved in the optimal range of human cell size 2–120 microns. The smallest pores were formed in scaffold Collagen/Complex/PRP. In contrast, porosity value for this scaffold moved higher than most other scaffolds. Scaffold with PRP had smaler average values of pore size than coresponding scaffold without PRP, except the Collagen/CaOC scaffold. On the other hand, porosity increased with the addition of PRP in all cases. The collagen-aditive scaffolds had higher porosity in the comparison with pure collagen, which decreased after cross-linking in followed order: Collagen/CaOC > Collagen/Chitosan > Collagen/Complex corresponding to porosity of 65.2 % > 59.2 % > 57.5 %, respectively. The porosity of samples increased for each with addition PRP.

The most samples did not have homogenous structure in whole volume. Transversal and longitudinal sections exhibited different locations in the volume of scaffold, which differ in structure, morphology and pore size distribution.

Though porosity affected the swelling behaviors of collagen sponges, the nature of additive plays more significant role, which demonstrated the lowest swelling ratio. The highest amounts of water absorbed pure collagen scaffold (98.67 %) in comparison with collagen sponges with additives. The swelling properties of collagen sponges modified with bioactive aditives decreased in followed order Collagen > Collagen/Complex > Collagen/Chitosan > Collagen/CaOC. Swelling ratios of collagen sponges enriched with PRP distincly decreased in comparison with sponges without PRP. The influence of crosslinking of the scaffolds with additives CaOC and complex was also studied using the swelling method. There was marked decline of swelling ratios at non-crosslinked samples of tens.

By stability test was confirmed the effect of the crosslinking agent, when non-crosslinked samples disintegrated first. The hydrolytic stability of non-crosslinked samples after a certain time increased in order Collagen/CaOC/PRP < Collagen/Complex/PRP < Collagen/CaOC < Collagen/Complex. The highest resistance of collagen sponges to hydrolytic degradation in water exhibited crosslinked samples and was not disintegrated after a period of 300 days.

The expected types of bond linkages presented in the samples were confirmed using infrared analysis. Pure collagen displays bands, which are related to the amide A, I, II and III bands of collagen, which are characteristic of the peptide bond.

As for pure collagen samples in the form of sponges prepared by lyophilization, characteristic bands were interpreted. Area and location of individual band maximum were changed according to the changes in the structure of collagen.

Characteristic bands of calcium oxycellulose can be assigned to bands which confirmed the presence of COO^- group, carboxyl group COOH, –OH group, C–H deformation vibration from secondary alcohol, –OH bending vibration, antisymetric C–O bridge stretching and CH_2–O–CH_2 stretching. Both COO^- and COOH, these functional groups of CaOC are not involved in collagen sponges modified with CaOC.

The spectrum of chitosan showed some typical bands which correspond to amides A, I, II, CH_2–O–CH_2 stretching in the pyranose ring, C–H deformation vibration from bounded and free secondary alcohols, C–O stretching from tertiary alcohol (bridge in the pyranose ring) and C–O stretching from primary alcohol. Pure chitosan and pure complex absorbed at wavenumbers, which are characteristic for the same vibrations. Pure complex sprectrum lacked the only band coresponding to Amid II.

Comparing the spectra of unmodified and modified scaffolds it is obvious that the major bands of mixtures had the same resulting position with small deviations in the order of units of cm^{-1}. Two intense bands that belong to stretching frequency of the –OH group and C=O stretching vibrations appeared in pure PRP spectrum. IR spektra of all samples enriched with PRP assigned with only one characteristic band around 1 395 cm^{-1} and the vibration belongs to >N–CH_3.

The results from DSC measurement indicated differences in the hydrothermal stability of collagen scaffolds depending on the type of additives and the degree of crosslinking. With increasing EDC/NHS-crosslinking, an increase in transition temperature was detected. For crosslinked Collagen/PRP sponge the highest transition temperature of 71.0 °C was detected. The addition of bioactive substance caused a decrease of transition temperature. In all cases, sample enrichment with PRP increased the transition temperature.

Influence of additive on the viability and proliferation of fibroblasts was measured by MTS assay and PicoGreen method in the 1st, 3rd, 7th, 10th and 14th day of experiment. The enrichment by PRP of collagen/additive sponges had very positive effect on cultivation of cells. The best cell viability according to MTS assay exhibited samples modified with bioactive additive and enriched with PRP. Scaffolds modified with CaOC and chitosan showed the best proliferation properties according to PicoGreen method. Unmodified scaffolds and scaffold modified with complex exhibited distinctly lower amount of absorbed PicoGreen.

Based on the obtained results, the collagen sponges involving bioactive additive could be used as scaffold for growing cells in systems with low mechanical loading. The collagen/additive sponges showed high porosity thus they could be used as scaffolds with potential application in biomedicine. In fine, the best material properties useful to the soft tissue scaffolds approved 0.5 % crosslinked collagen sponge modified with chitosan or chitin/chitosan-glucan complex, both enriched with PRP due to its convenient morphology, stability and biological properties. Although the Collagen/CaOC/PRP scaffold exhibited high

cell viability as well, it changed the structure during the swelling, where the scaffold did not keep shape and was too soft.

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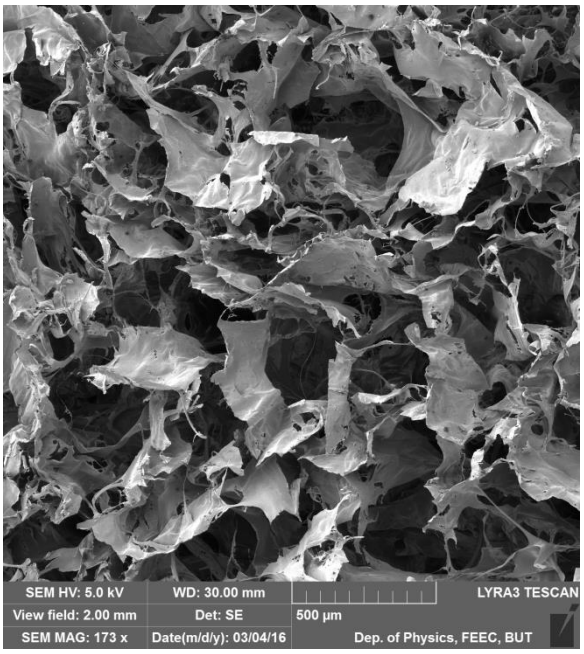
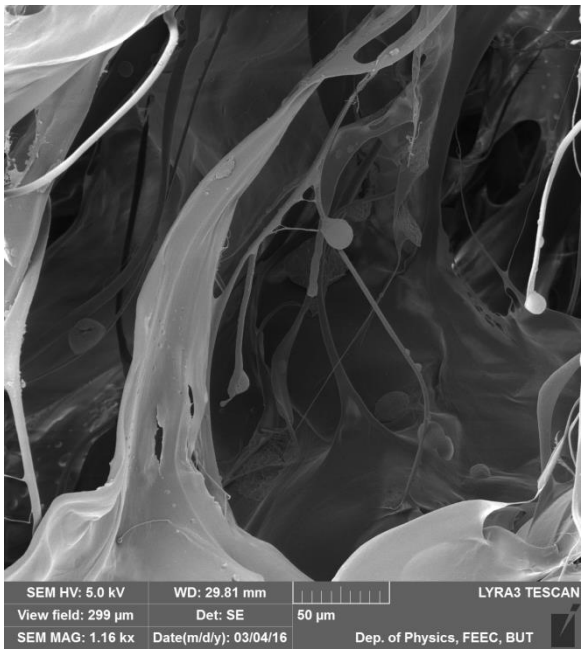
7 LIST OF APPENDIXES

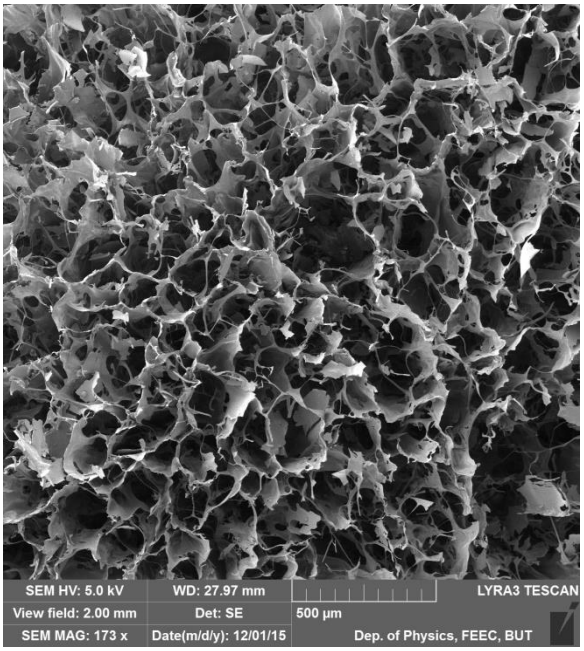
8 APENDIXES

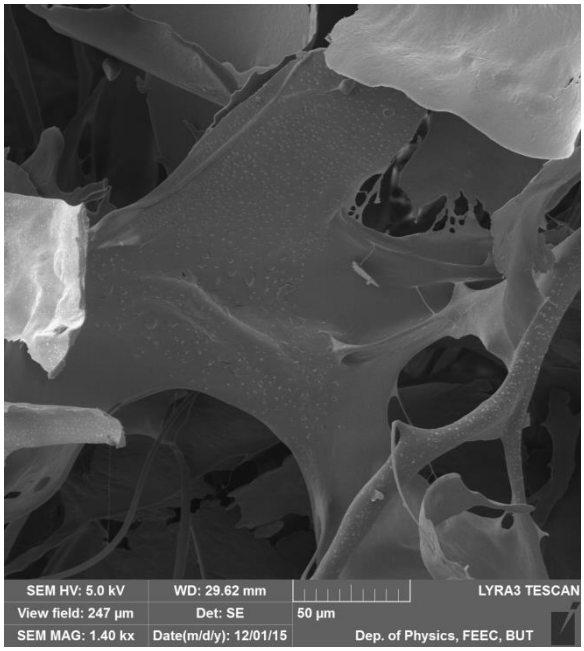
a) Collagen_crosslinked





b) Collagen/PRP_crosslinked





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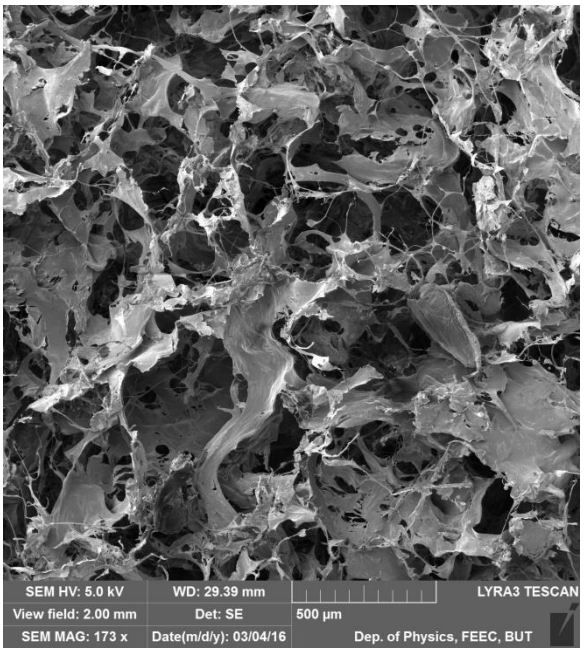

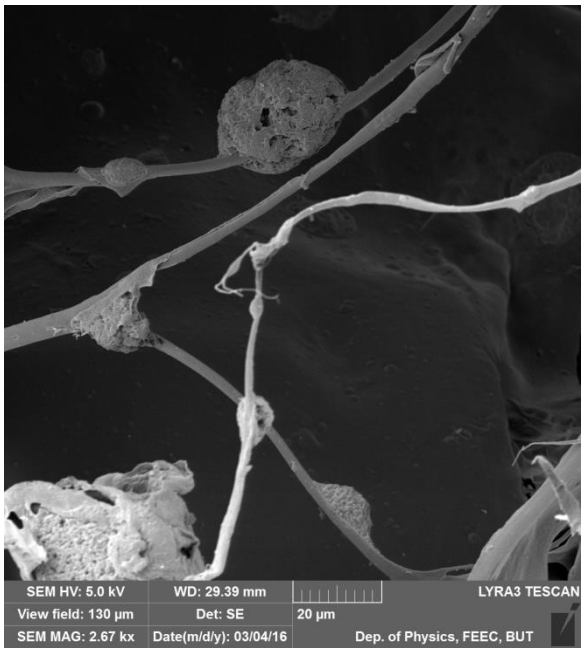

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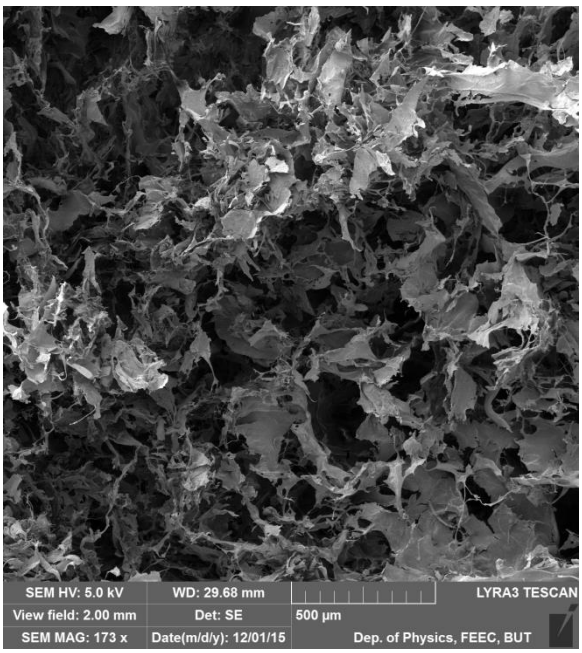
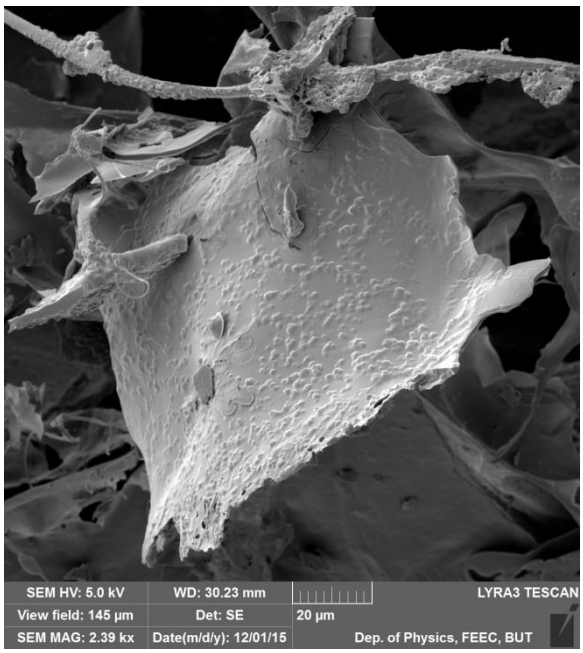
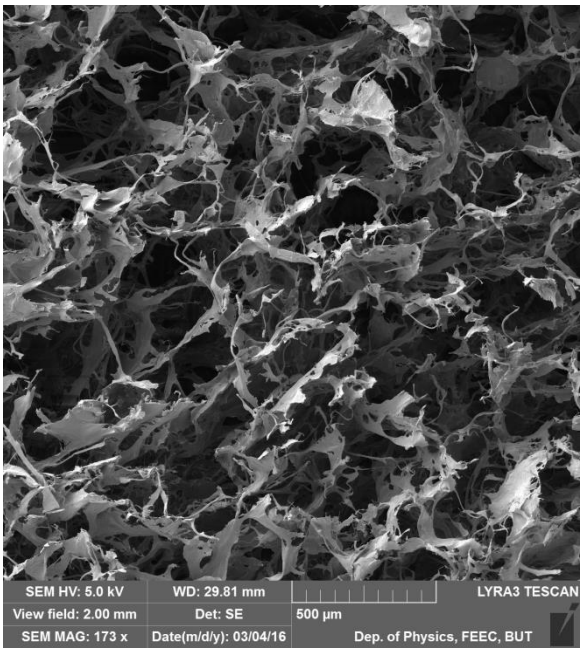
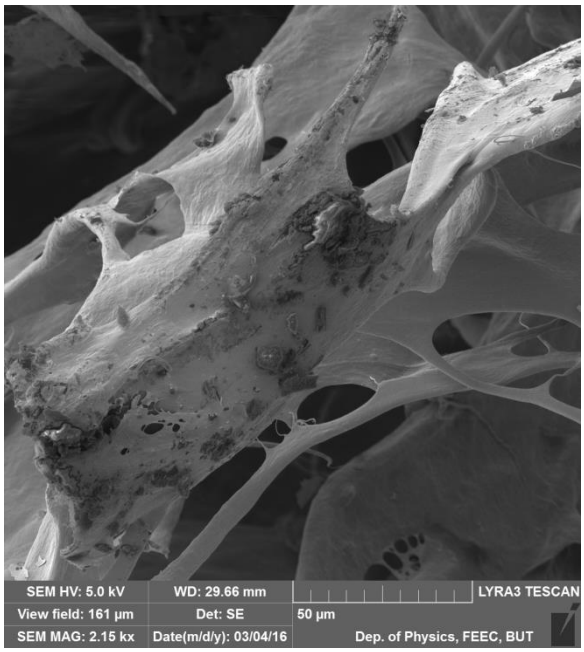




e) Collagen/CaOC_crosslinked





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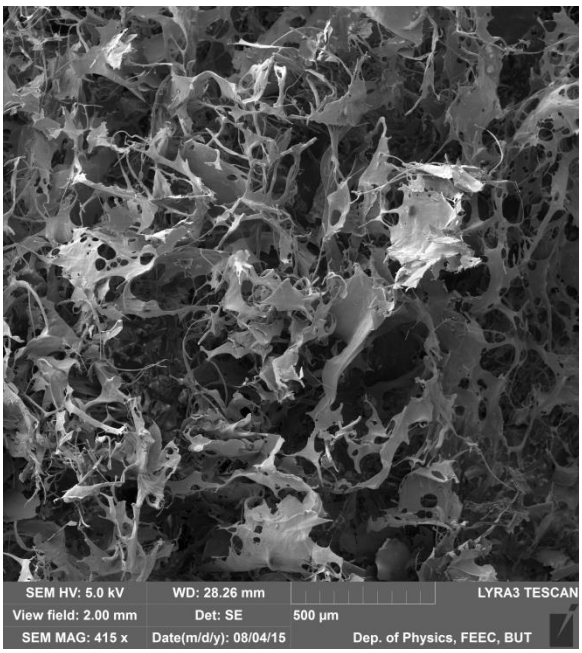

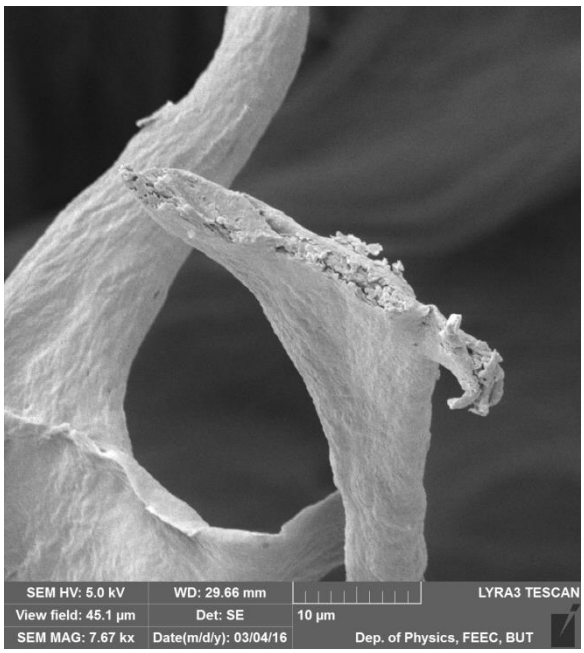

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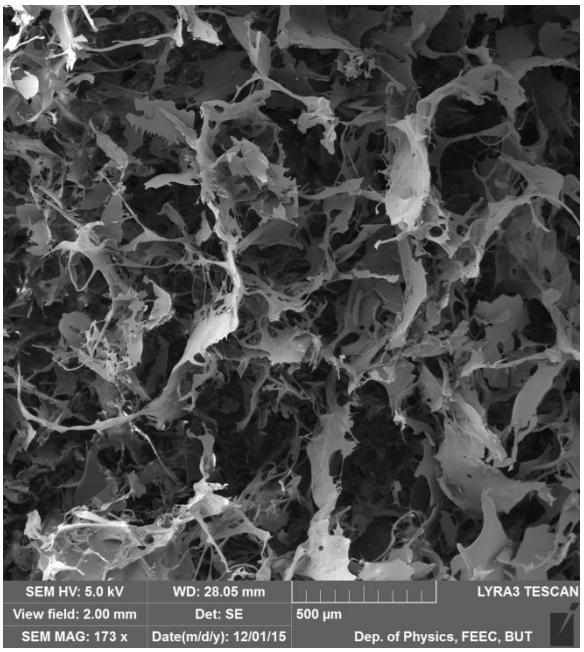
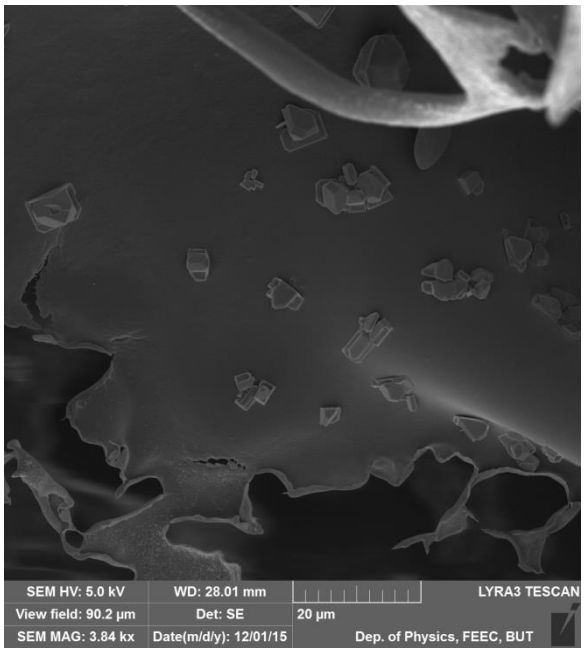
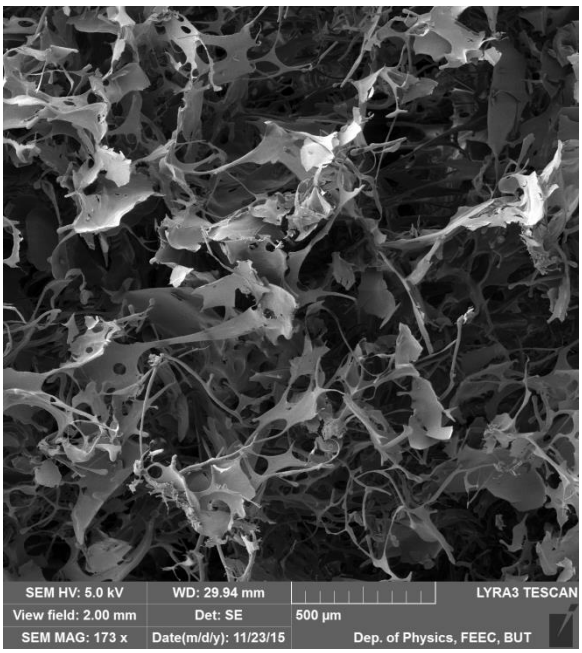
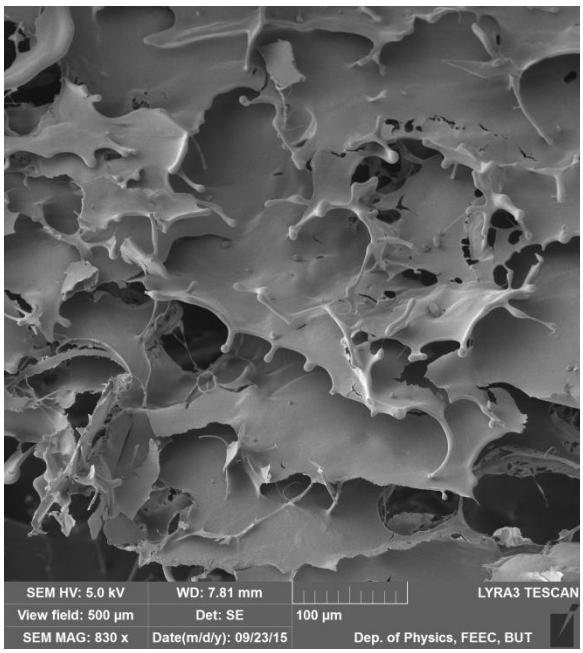




f*) Collagen/CaOC/PRP_non-crosslinked





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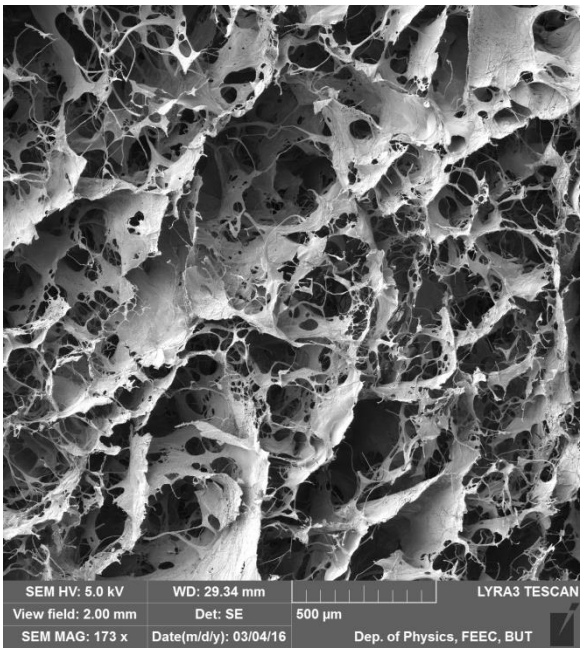
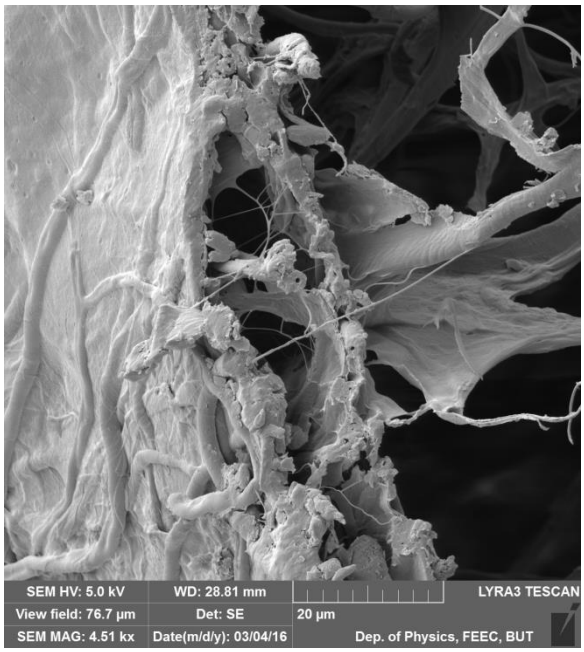
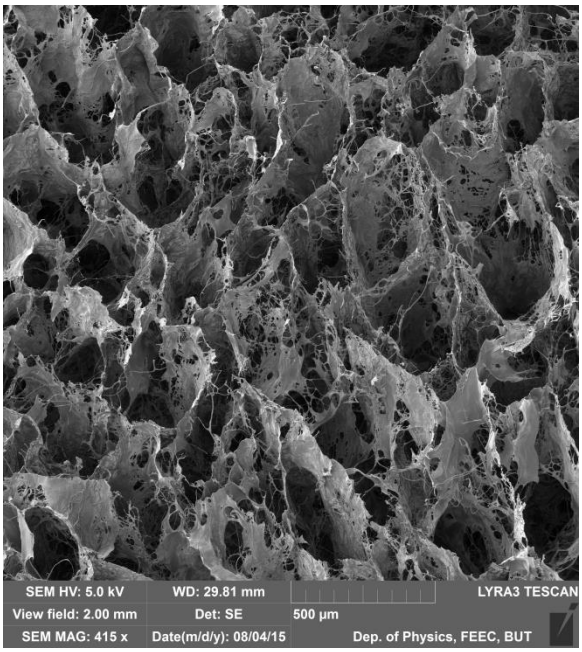
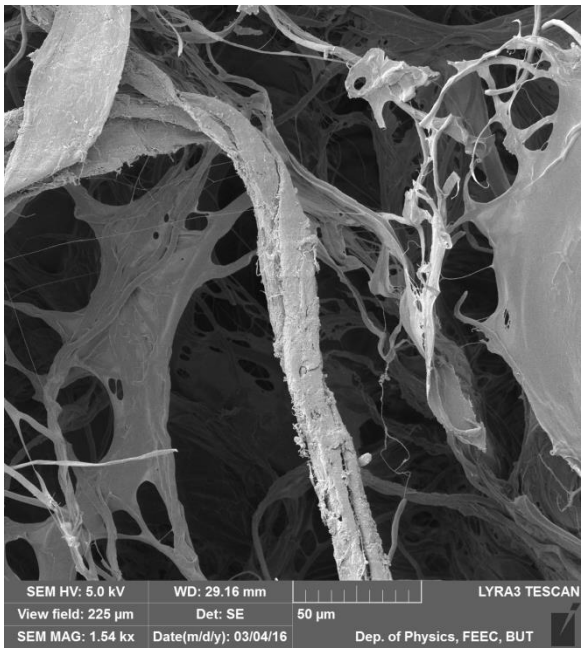




g*) Collagen/Complex_non-crosslinked





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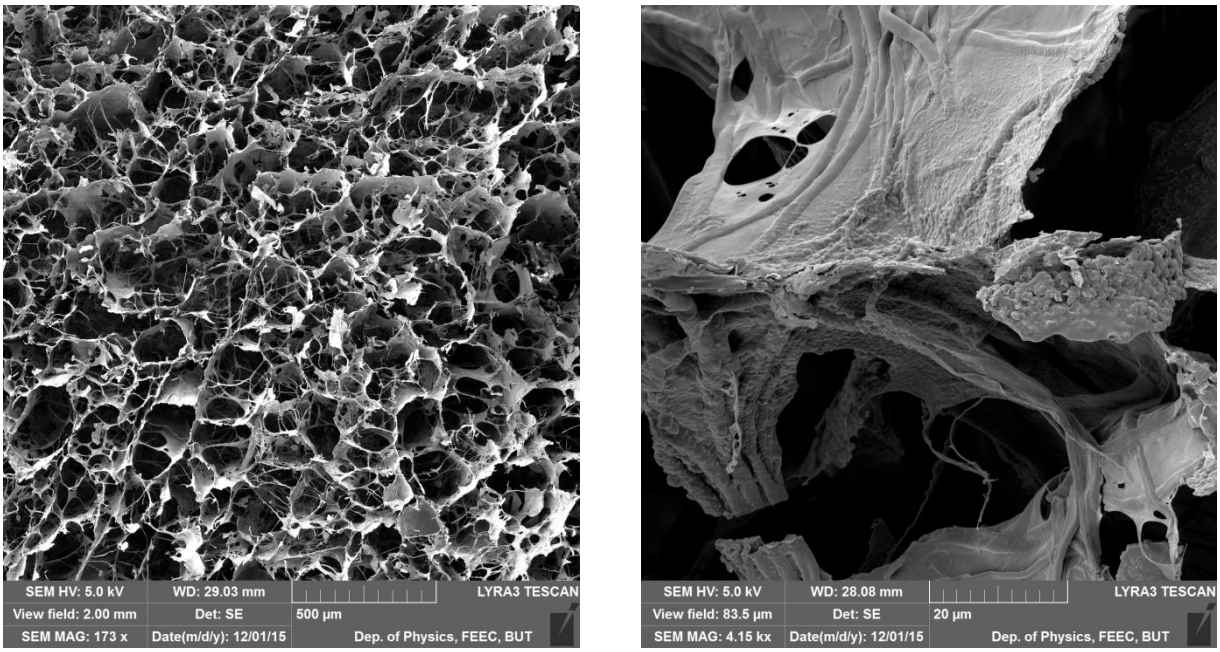
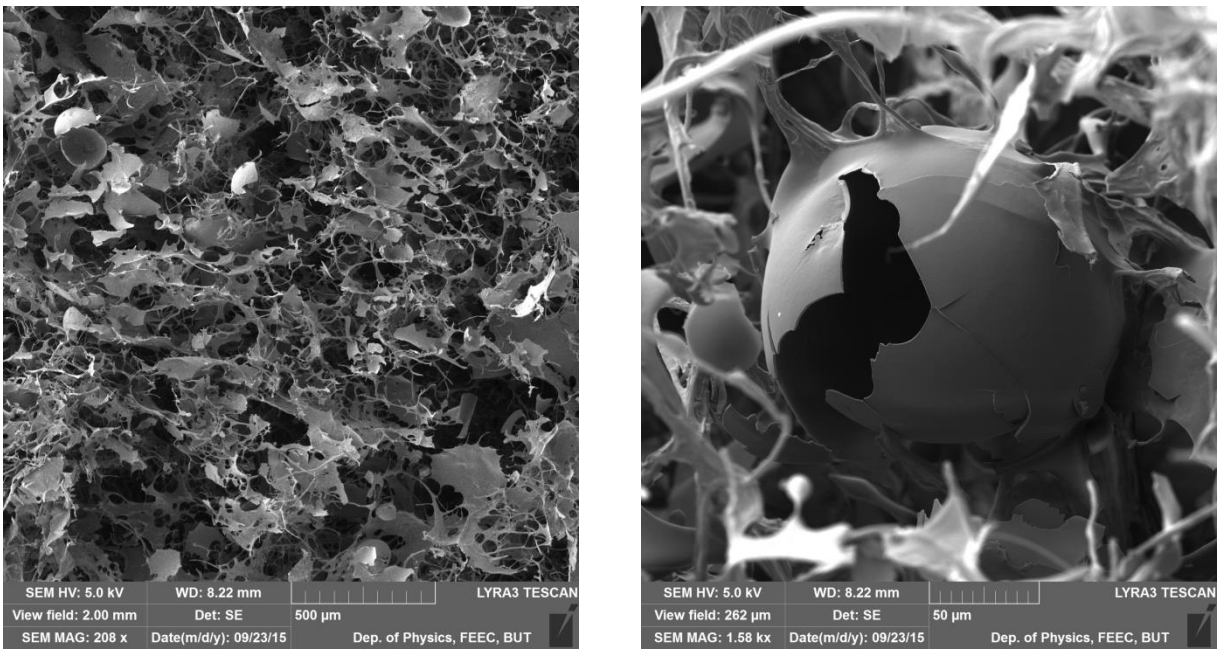
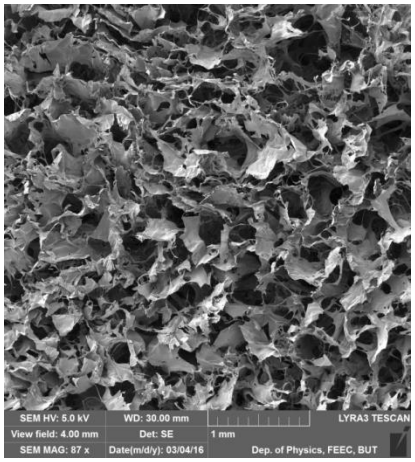


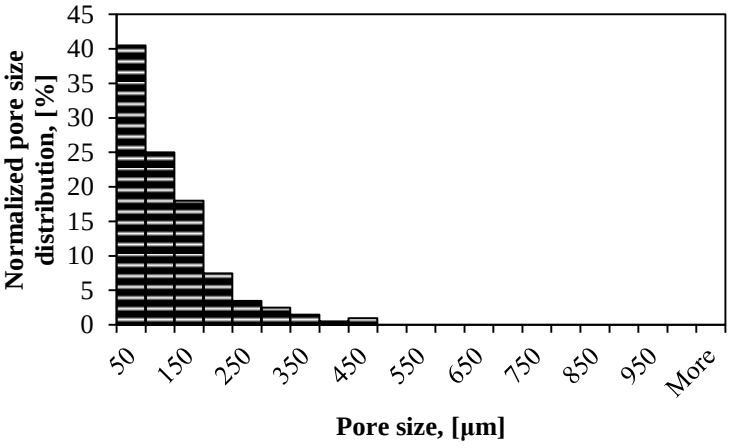
h*) Complex_PRP_non-crosslinked

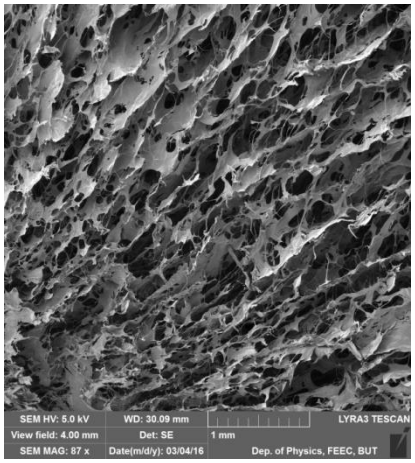


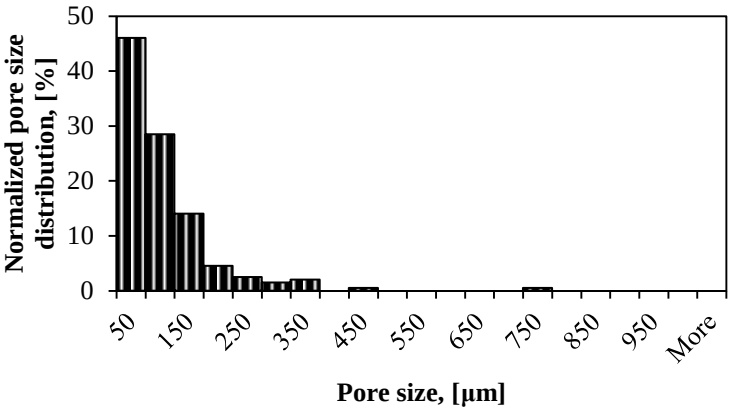
*Appendix 1: Morphologies and details in structure of all prepared crosslinked (_C) and non-crosslinked (*_N) collagen sponges with various additives in first and second serie observed by SEM: a) Collagen_C, b) Collagen/PRP_C, c) Collagen/Chitosan_C, d) Collagen/Chitosan/PRP_C, e) Collagen/CaOC_C, e*) Collagen/CaOC_N, f) Collagen/CaOC/PRP_C, f*) Collagen/CaOC/PRP_N, g) Collagen/Complex_C, g*) Collagen/Complex_N h) Collagen/Complex/PRP_C, h*) Collagen/Complex/PRP_N.*

a) Collagen









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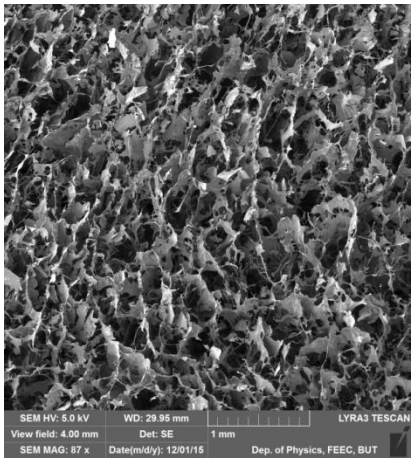
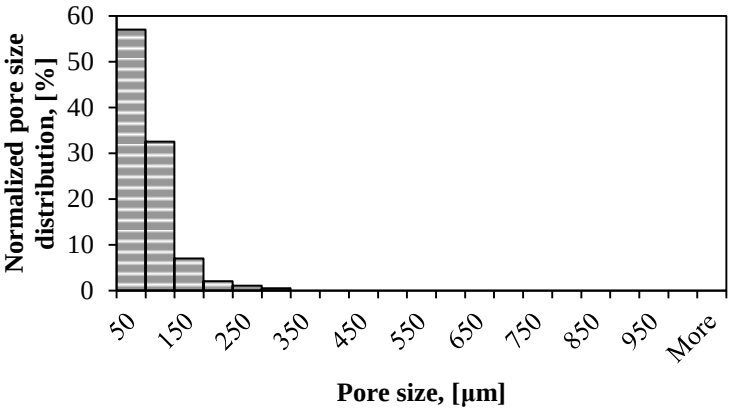
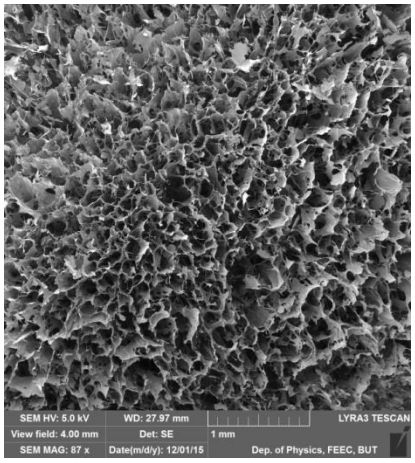
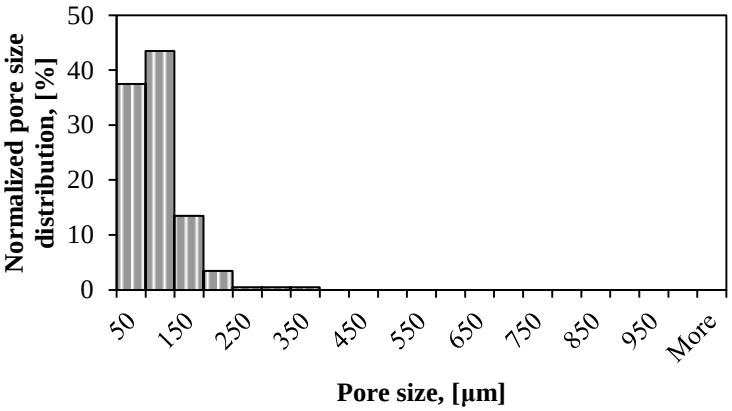
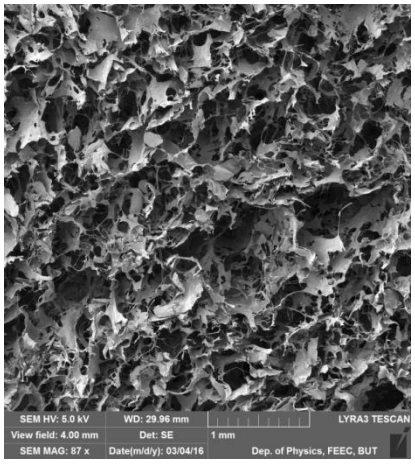


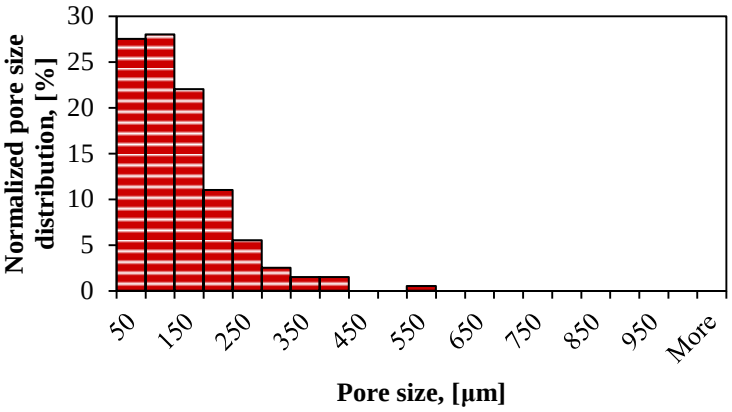


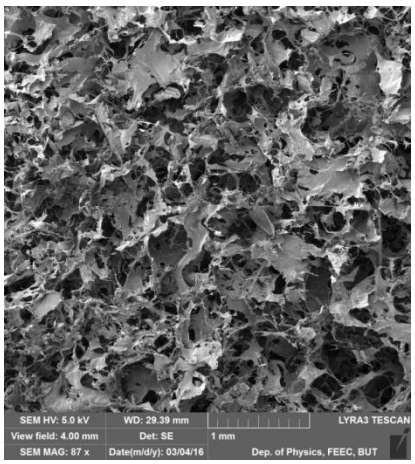


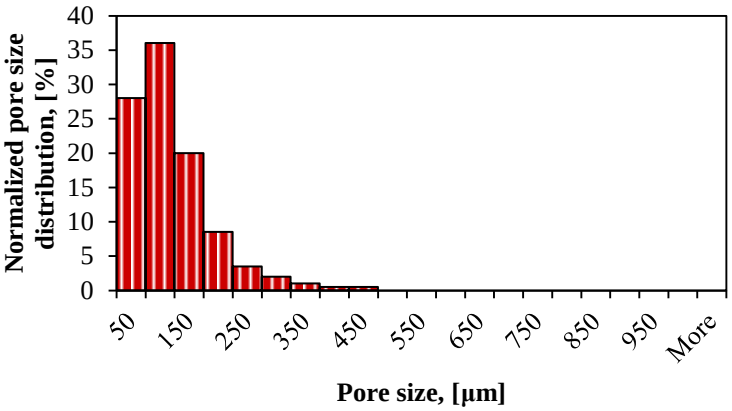


c) Collagen/Chitosan









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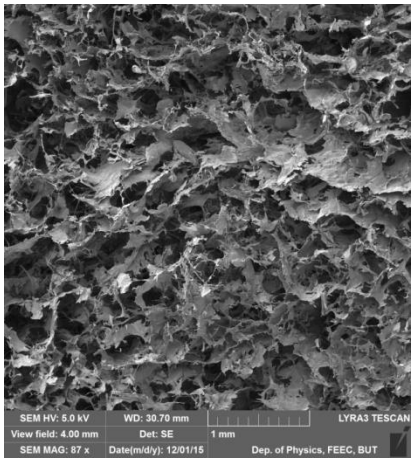
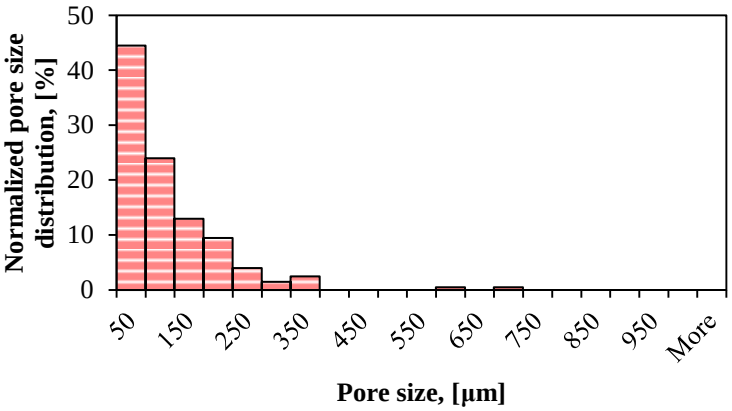
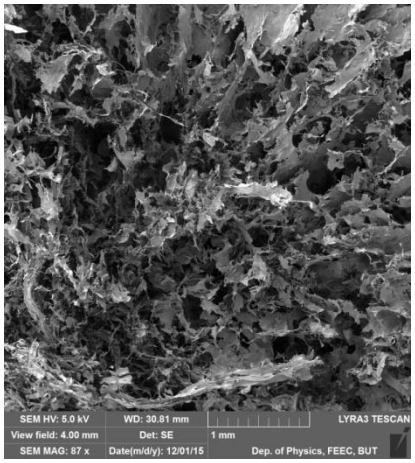
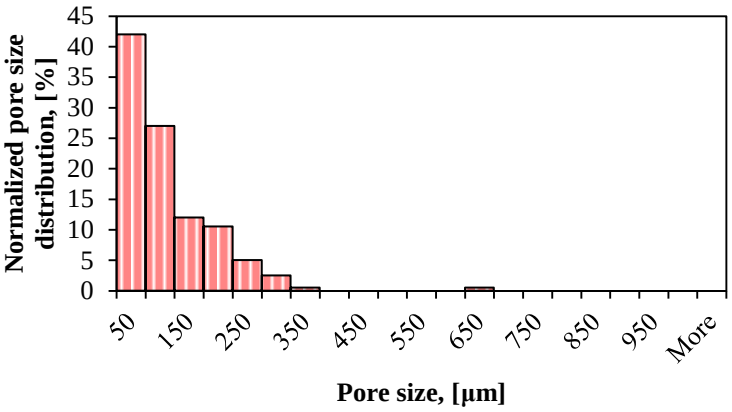
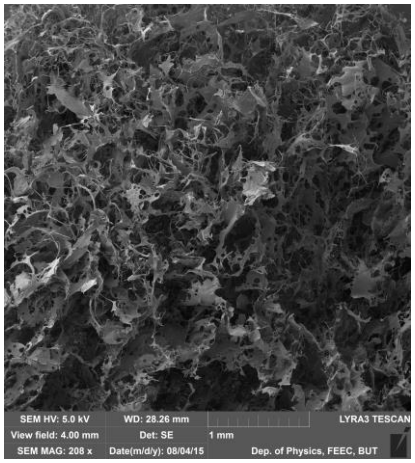


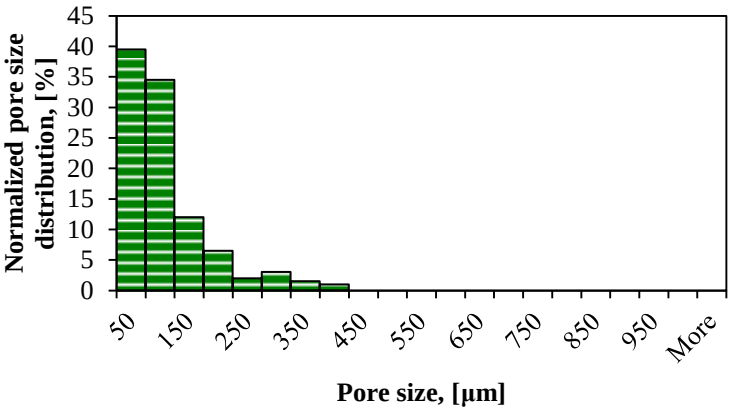


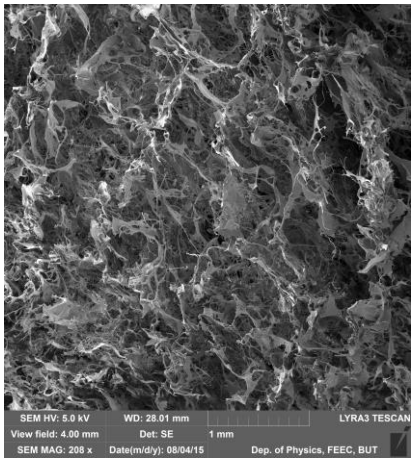


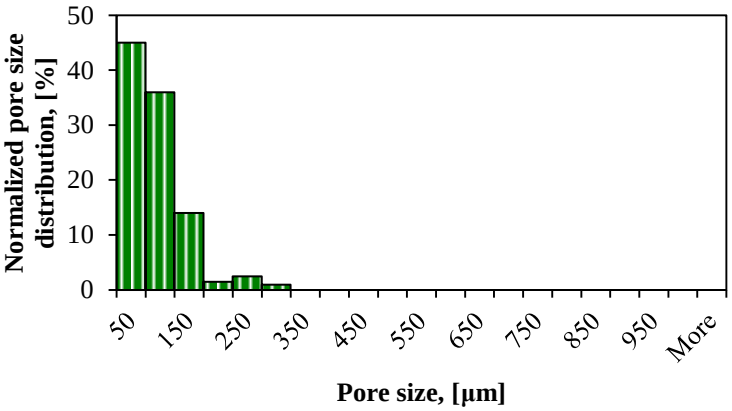


e) Collagen/CaOC

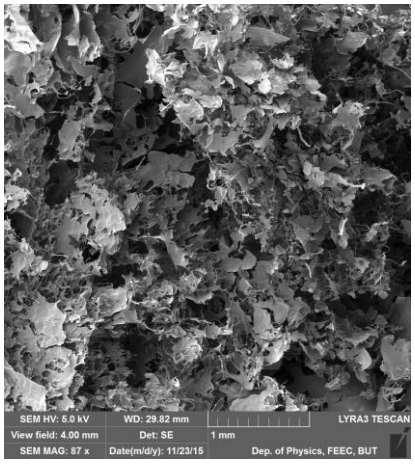


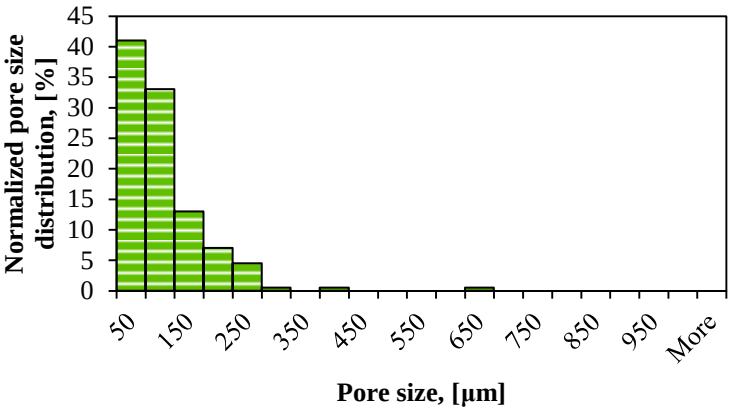


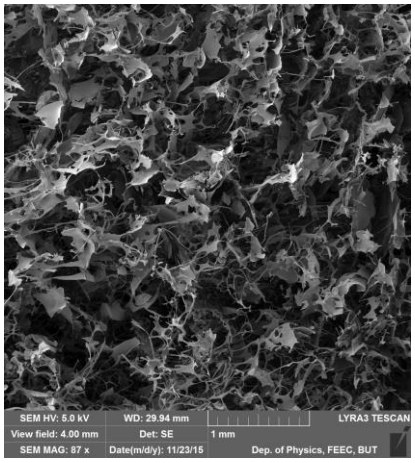


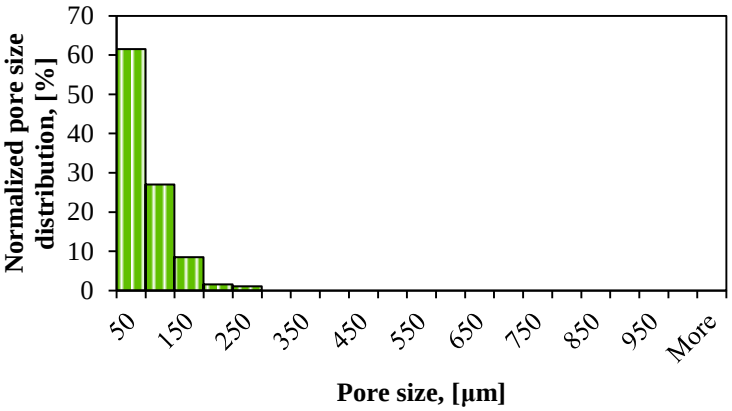


f) Collagen/CaOC/PRP

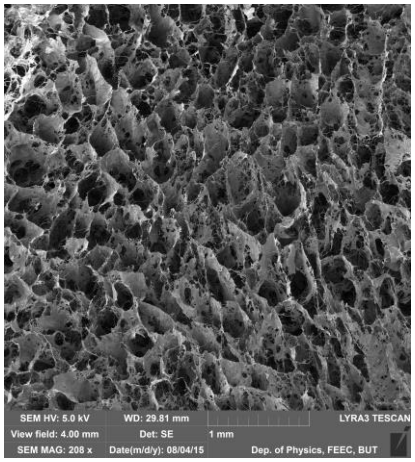


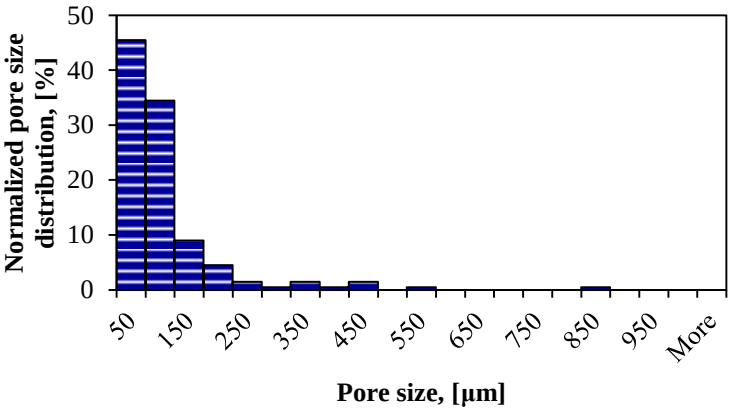


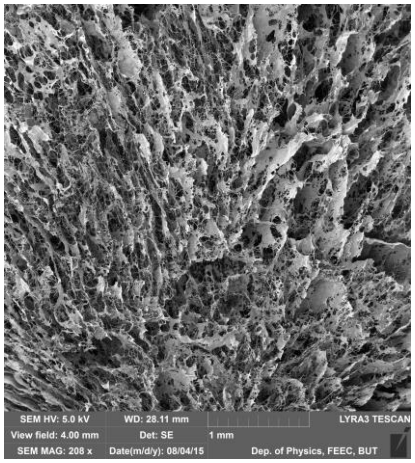


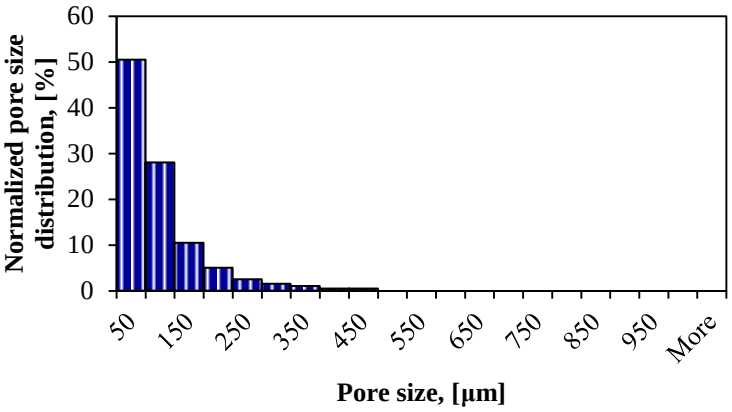


g) Collagen/Complex

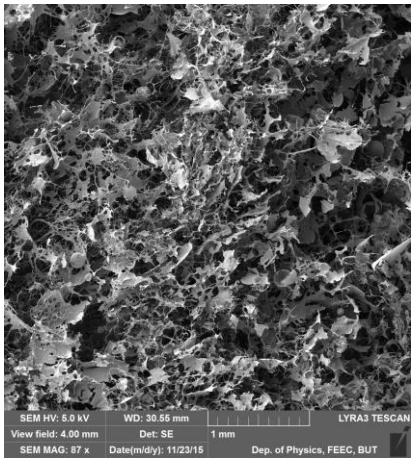


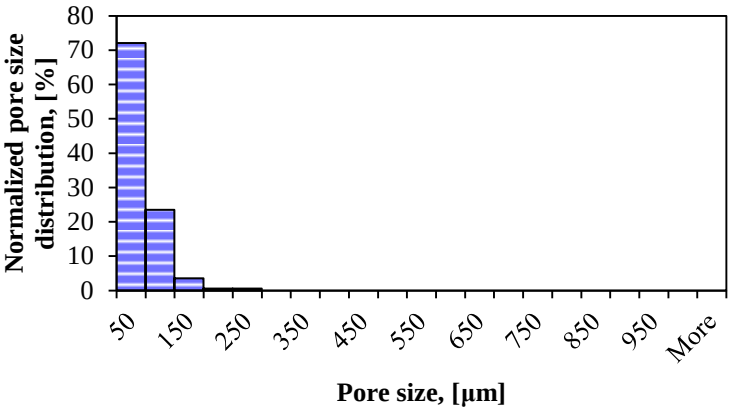


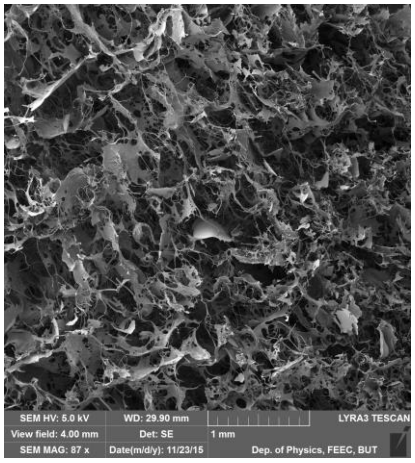


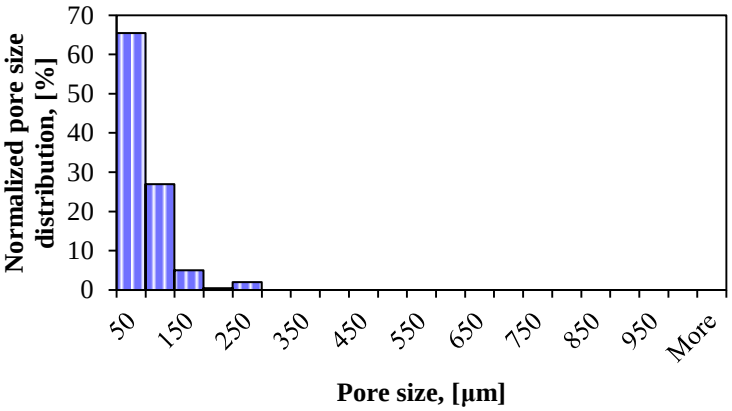


h) Collagen/Complex/PRP



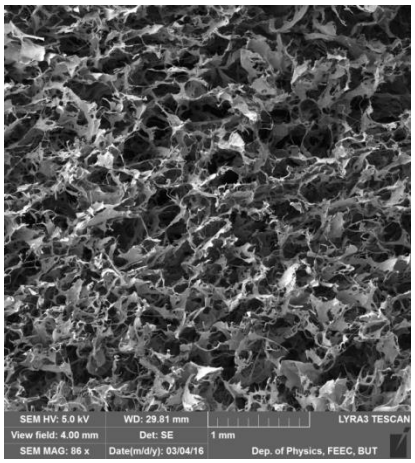


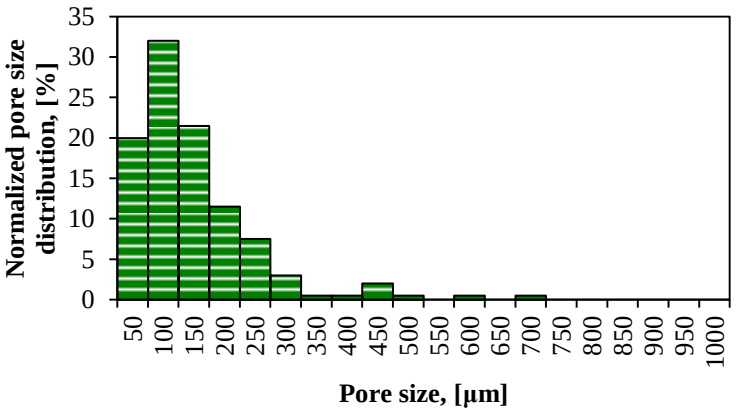


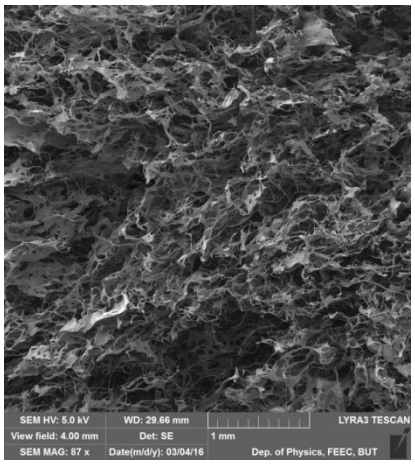


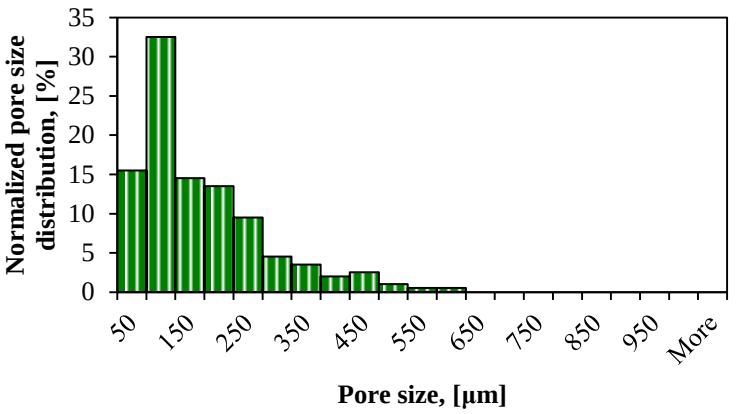
Appendix 2: Pore size distributions calculated for all prepared collagen sponges with various additives in second serie (all crosslinked): a) Collagen, b) Collagen/PRP, c) Collagen/Chitosan, d) Collagen/Chitosan/PRP, e) Collagen/CaOC, f) Collagen/CaOC/PRP, g) Collagen/Complex. h) Collagen/Complex/PRP, Up – cross section, down – longitudinal section.

e*) Collagen/CaOC

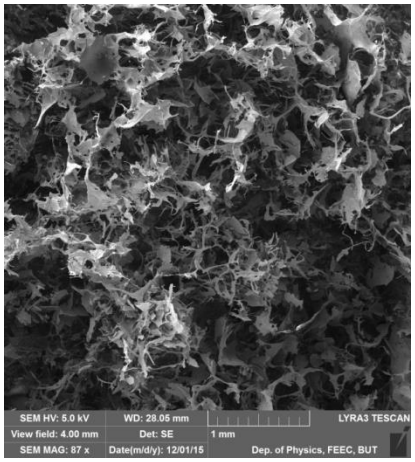


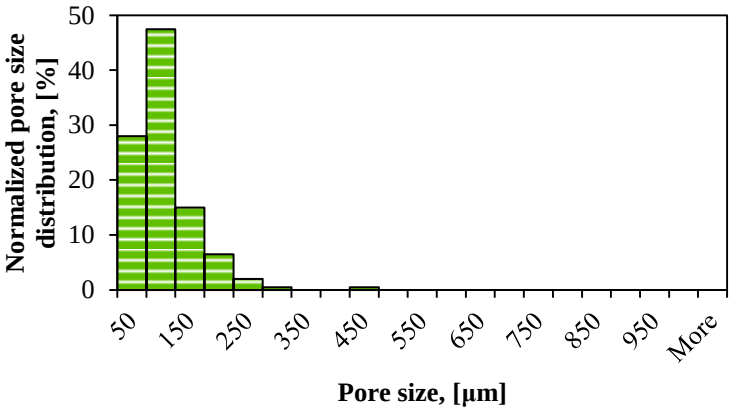


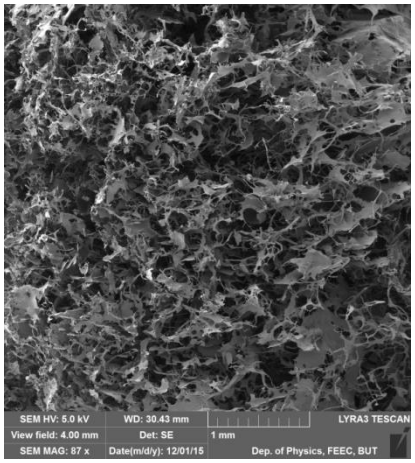


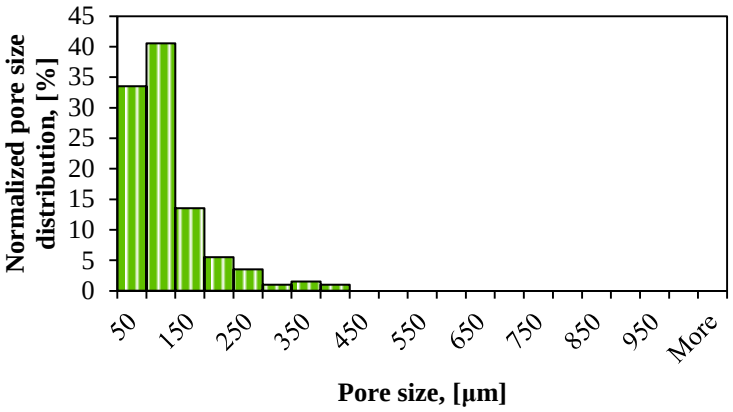


f*) Collagen/CaOC/PRP

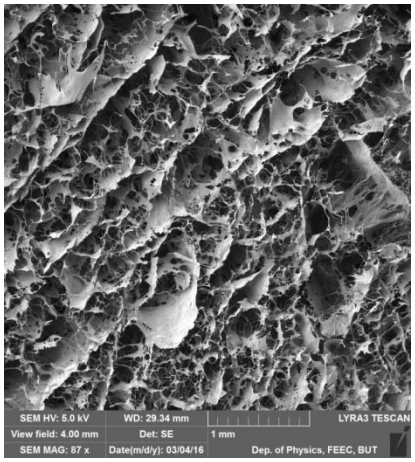


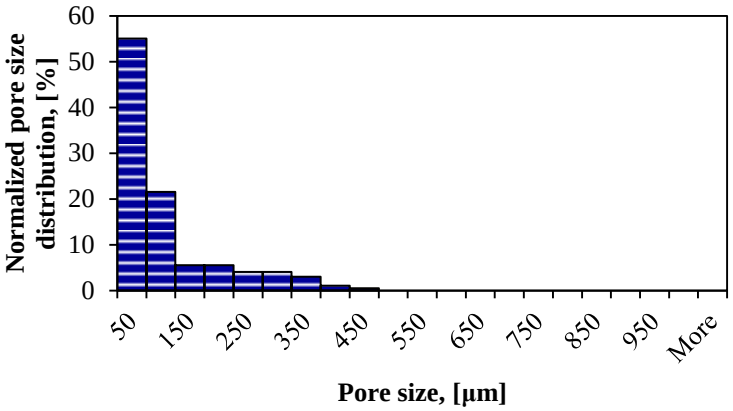


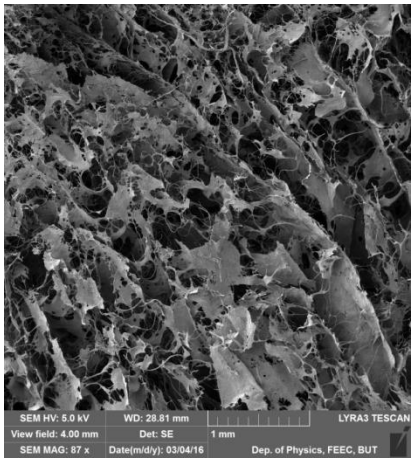


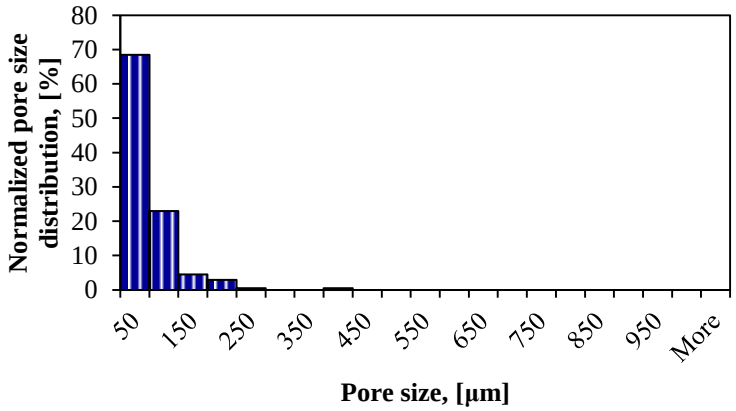


g*) Collagen/Complex

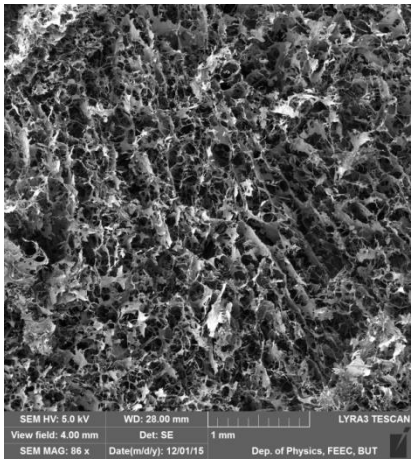


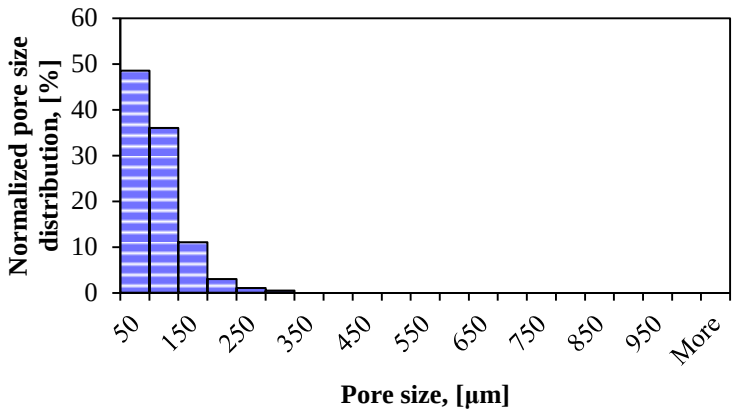


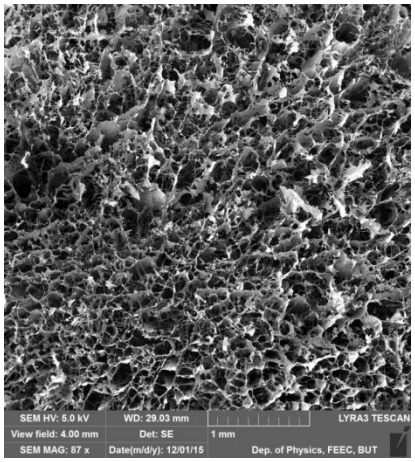


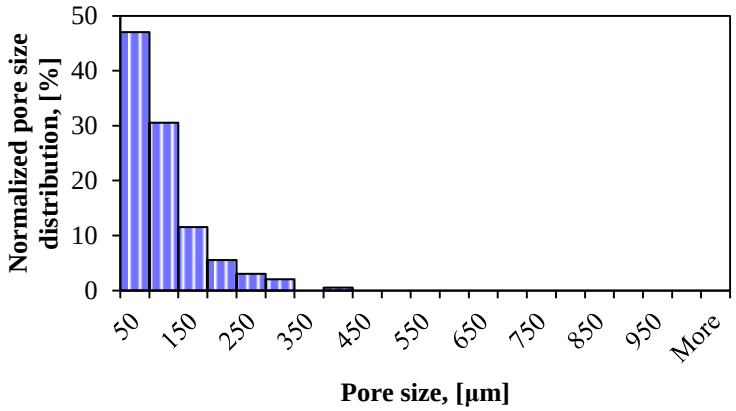


h*) Collagen/Complex/PRP

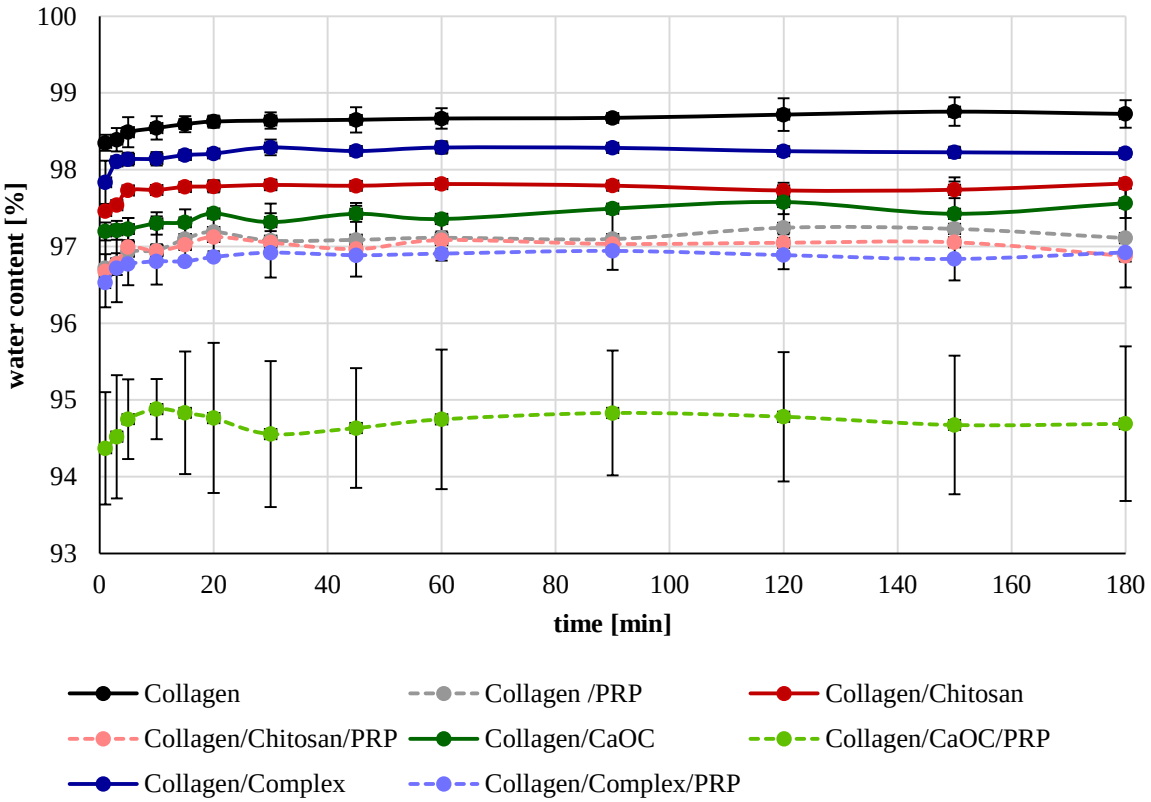








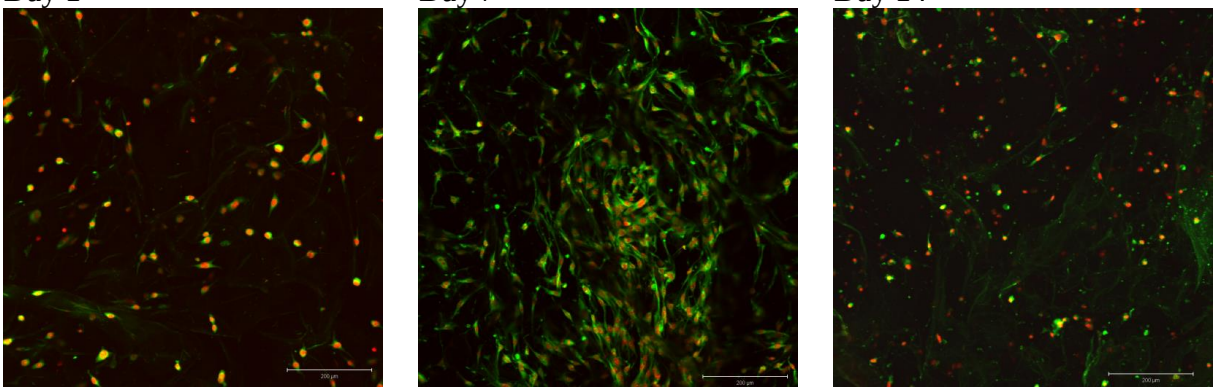
Appendix 3: Pore size distributions calculated for non-crosslinked collagen sponges with additives CaOC and Complex in first serie: e) Collagen/CaOC, f*) Collagen/CaOC/PRP g*) Collagen/Complex, h*) Collagen/Complex/PRP. Up – cross section. down – longitudinal section.*



Appendix 4: The dependence of water content on time for collagen scaffolds prepared in the second serie (all crosslinked).

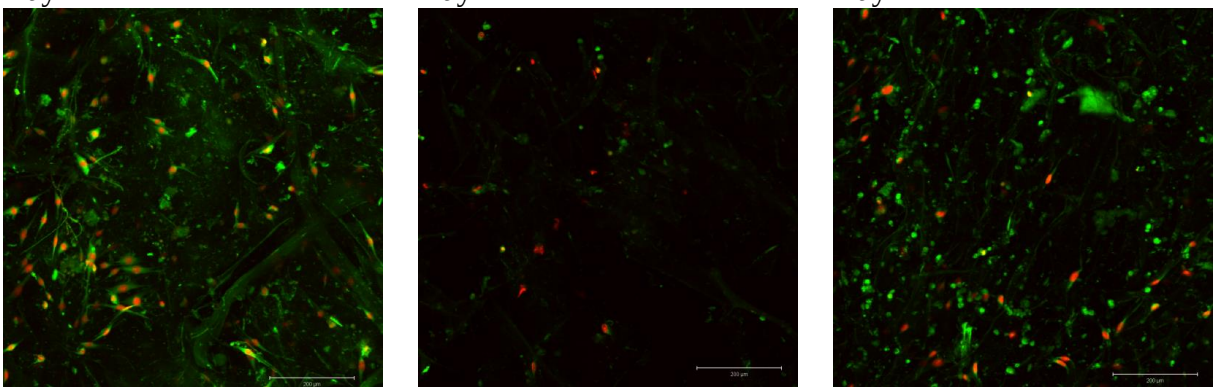
A)

Day 1 Day 7 Day 14



B)

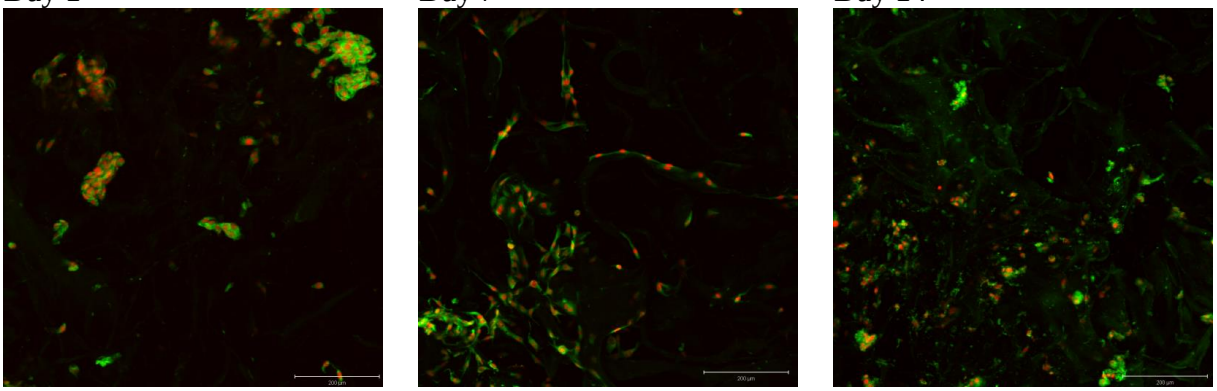
Day 1 Day 7 Day 14



Appendix 5: Adhesion and proliferation of the 3T3 fibroblasts on the A) Collagen and B) Collagen/PRP scaffold, $DiOC_6$/Propidium iodide staining, 200 micron scale.

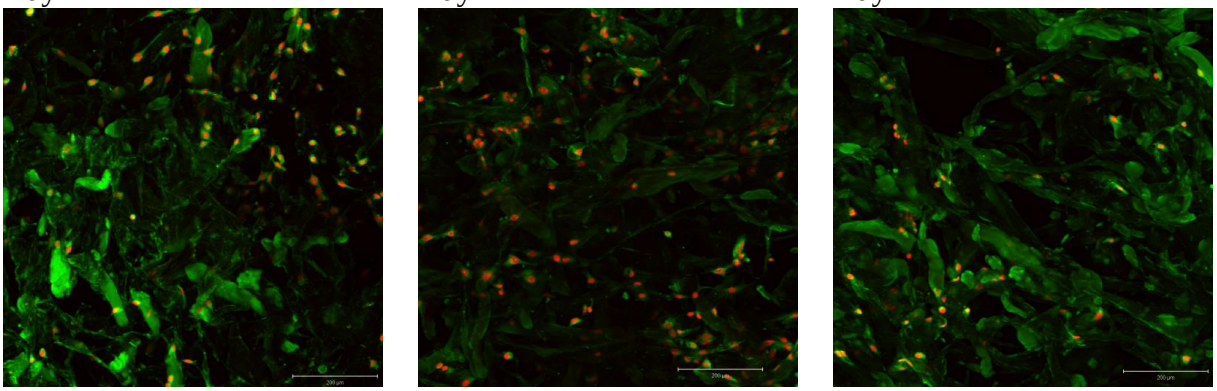
A)

Day 1 Day 7 Day 14



B)

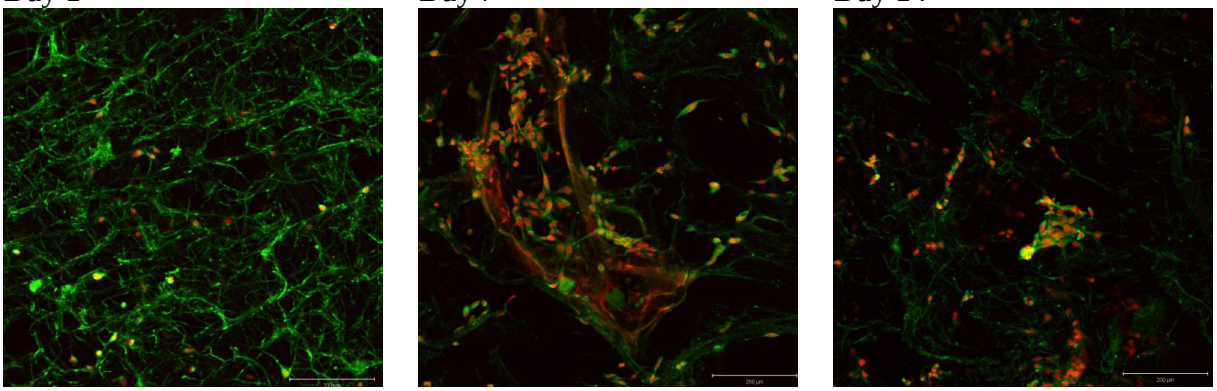
Day 1 Day 7 Day 14



Appendix 6: Adhesion and proliferation of the 3T3 fibroblasts on the A) Collagen/CaOC and B) Collagen/CaOC/PRP scaffold, $DiOC_6$/Propidium iodide staining, 200 micron scale.

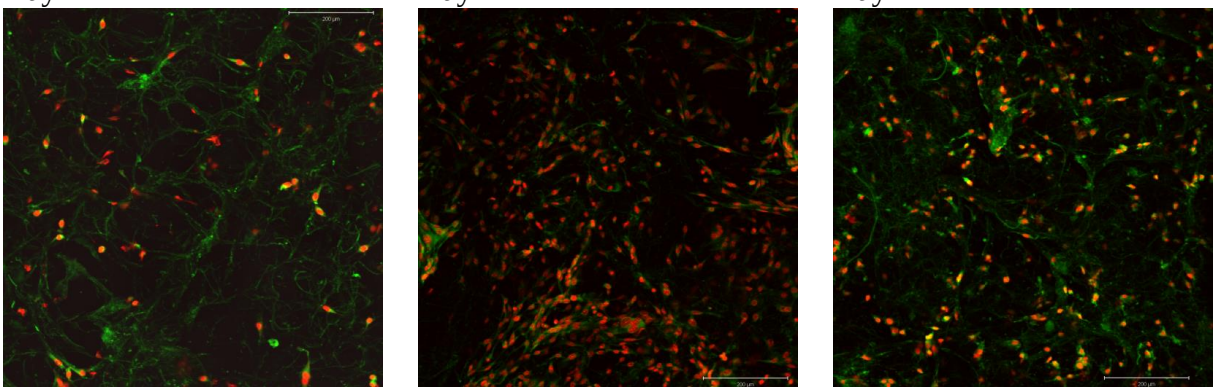
A)

Day 1 Day 7 Day 14



B)

Day 1 Day 7 Day 14



Appendix 7: Adhesion and proliferation of the 3T3 fibroblasts on the A) Collagen/Complex and B) Collagen/Complex/PRP scaffold, $DiOC_6$/Propidium iodide staining, 200 micron scale.