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ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

FOTOSTABILIZATION OF BIOPOLYMERIC MATERIALS FOR REGENERATIVE MEDICINE
FOTOSTABILIZACE BIOPOLYMERNÍCH MATERIÁLŮ PRO REGENERATIVNÍ MEDICÍNU

BACHELOR'S THESIS
BAKALÁŘSKÁ PRÁCE

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Fotostabilization of biopolymeric materials for regenerative medicine

Bachelor's Thesis:

1) Literature review concerning stabilisation of biopolymers for the needs of regenerative medicine.
2) Prepraration of biopolymeric materials and their stabilisation using short wavelength radiation.
3) Comparison of physicochemical properties of photostabilized materials with standardly–used chemically crosslinked materials.
4) Evaluation and interpretation of results.
5) Conclusion.

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ABSTRACT

Non-stabilized biopolymers often show poor mechanical properties. The stabilization and improvement of these properties could be accomplished by crosslinking, but the traditionally used crosslinking agents are rather cytotoxic and harmful for the human body. The aim of this work is to find a suitable non-toxic crosslinking method that would be efficient enough, but at the same time would not represent danger to any organism. The crosslinking method developed in the presented bachelor thesis should be comparatively efficient as traditionally used methods (e.g. chemical carbodiimide-based crosslinking) as well as potentially less cytotoxic by use of naturally derived substances. The proposed alternative crosslinking method should improve the strength and stability of the crosslinked biopolymers and enhance their biological functionality. Specifically, the presented work deals with the stabilization of collagen scaffolds using vitamin important for human metabolism followed by short- and long-wavelength irradiation. The theoretical part contains a general overview of possible non-toxic crosslinking agents, describes the structure of collagen and characterizes its properties and with the properties of used vitamin as a crosslinking agent. The experimental part deals with the preparation and crosslinking optimization of collagen scaffolds. Swelling, degradation and morphology of scaffolds as well as collagen secondary structure were evaluated. As a result, sufficiently strong crosslinked scaffolds were obtained.

KEYWORDS

Regenerative medicine, crosslinking agents, collagen scaffolds, stabilization of biopolymers, vitamins, morphology

ABSTRAKT

Nestabilizované biopolyméry často vykazujú zlé mechanické vlastnosti. Stabilizáciu a zlepšenie týchto vlastností je možné dosiahnuť sieťovaním, ale tradične používané sieťovacie činidlá sú pre ľudské telo často cytotoxické a škodlivé. Cieľom tejto práce je nájsť vhodnú netoxickú sieťovaciu metódu, ktorá by bola dostatočne účinná, ale zároveň by nepredstavovala nebezpečenstvo pre žiadny organizmus. Metóda sieťovania vyvinutá v predloženej bakalárskej práci by mala byť porovnateľne účinná ako tradične používané metódy (napr. sieťovanie na báze chemického karbodiimidu) a tiež potenciálne menej cytotoxické pri použití prírodných látok. Navrhovaná alternatívna metóda sieťovania by mala zlepšiť pevnosť a stabilitu sieťovaných biopolymérov a zvýšiť ich biologickú funkčnosť. Predkladaná práca sa konkrétne zaoberá stabilizáciou kolagénových skafoldov pomocou vitamínu dôležitého pre ľudský metabolizmus a následným ožarovaním krátkou a dlhou vlnovou dĺžkou. Teoretická časť obsahuje všeobecný prehľad možných netoxických sieťovacích činidiel, popisuje štruktúru kolagénu a charakterizuje jeho vlastnosti a vlastnosti použitého vitamínu ako sieťovadla. Experimentálna časť sa zaoberá prípravou a optimalizáciou sieťovania kolagénových skafoldov. Skúmalo sa botnanie, degradácia a morfológia skafoldov, ako aj sekundárna štruktúra kolagénu. Výsledkom bolo získanie dostatočne silných sieťovaných skafoldov.

KĽÚČOVÉ SLOVÁ

Regenertívna medicína, sieťovadlá, kolagénové skafoldy, stabilizácia biopolymérov, vitamíny, morfológia

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DECLARATION

I declare that the bachelor thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the bachelor thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student's signature

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CONTENT

1 INTRODUCTION

After many years of research, currently, the regenerative medicine is an emerging multidisciplinary field representing a valuable tool to cure severe clinical conditions. Regenerative medicine and cell therapy offer various treatment options, which are rarely considered in daily clinical settings. Collagen-based scaffolds are frequently used in regenerative medicine mainly as a scaffolding material for cells. Collagen, a protein consisting of three polypeptide chains assembled into a triple helix conformation, is an important biopolymer used in tissue engineering because of its biocompatibility, low antigenicity, biodegradability and cross-linking ability, which help controlling its mechanical properties and stability in the hydrated environment. Collagen-based materials represent a promising alternative for skin tissue replacement, but to do so they need to be stabilized usually by crosslinking. Crosslinking is a process during which the polymers form additional intra- and intermolecular bonds. This process enhances the stability and improves the mechanical and biological properties of the produced scaffolds. These scaffolds are later employed in regenerative medicine by implantation into the defected place. An ideal biomaterial for regenerative medicine should be non-toxic, biocompatible and promoting cellular interactions to tissue development, with adequate mechanical and physical properties. The produced scaffolds should not demonstrate inflammatory or immunological reactions. However, many crosslinking agents are cytotoxic and need to be washed out after the crosslinking process, therefore it makes the production of the scaffolds more expensive and time-consuming. The scientific community is thus focusing on finding non-toxic crosslinking agents that would be naturally derived with the same efficiency as its toxic predecessors. One of the possibilities could be vitamin B_2 known as riboflavin in combination with UV irradiation, due to its light instability. Moreover, riboflavin is important vitamin for the both animal and human metabolism thus exhibiting great biocompatibility and no signs of cytotoxicity.

2 THE STATE OF THE ART

2.1 Regenerative Medicine: An Approach to Wound Healing

Regenerative medicine (RM) has come a long way in many years of research and now it starts to represent a valuable implement to cure severe injuries and diseases. Regenerative medicine, tissue engineering (TE), and other related research fields offer various treatment options in healthcare. One of the aims of RM is to design different tissues and organs (e.g. bladder, hearth, nerve, ovary, skin, etc.). However, only some of these organs have been successfully implanted in humans, a lot of them remain still in the pre-clinical phase. There is still a long way to go before RM replace the common ways of traditional medicine.[1]

The fundamental elements of RM and TE are biomaterials. The ideal biomaterial for RM and TE should be non-toxic and biocompatible. These biomaterials should support cellular interactions for proper tissue development and have appropriate physical and mechanical properties.[2] Biomaterials are often divided into three main groups – metal-based, ceramic-based and polymer-based. Application of these materials depend on the type of the reconstructed tissue. Metal-based and ceramic-based materials are frequently used in regeneration of hard tissue – bone and tooth tissue. Application of biomaterials based on polymers is more diverse. Polymer-based materials are suitable for fabrication of soft tissue substitutes for tendons, ligaments, cartilage, skin, fibrous tissues, blood vessels, though they can find their application in hard tissue substitutes as well.[3] Polymer-based biomaterials can be divided based on the origin of the polymer. Polymers can be either extracted from the natural origin, often referred as biopolymers, or artificially synthetized, often referred as synthetic polymers.[2]

- *Biopolymers*. Naturally derived biopolymers have a lot of similarities with biological macromolecules, which are metabolized in the physiological environment of the human body. These biopolymers have an advantage to not cause inflammatory or immunological reactions and not being cytotoxic. Having a natural origin, these polymers are fully degradable enzymatically or hydrolytically with non-toxic degradation products. However, their mechanical performance in physiological environment is poor compared to other biomaterials and the structure is in need of further stabilization. Moreover, naturally derived biopolymers are prone to be exposed to the external environment during their production, which can cause microbial or heavy metal contamination. The contamination is resolved using preservatives, which slower down the processing of biopolymers and hamper the mass production for medical use. Natural polymers include proteins such as collagen, gelatin, elastin, actin, keratin, silk fibroin and albumin. A highly used group of natural polymers are also polymeric saccharides and their derivatives; chitin, cellulose, alginate, carrageenan, natural gumsand hyaluronic acid are the most used ones. [2][4]
- *Synthetic polymers*. Artificially acquired polymers are easily processable into different

sizes and shapes. Their physiochemical properties (crystallinity, molecular weight, etc.) can be easily modified by incorporating functional groups, selecting different monomers, and alternating reaction conditions. Overall, they can be designed to fulfill specific needs. *In vivo*, synthetic polymers undergo hydrolytic degradation which can lead to toxic intermediates of degradation. Moreover, products of degradation are often responsible for a local decrease of pH inducing a local inflammatory reaction. The most used synthetic polymers include biodegradable polyesters like poly(glycolic) acid, poly(lactic) acid, copolymers above-mentioned, poly(ε-caprolactone), poly(hydroxy alkanoate) and e.g. poly(dioxanone), poly(cyano acrylate) as well as some hydrophilic bioresorbable polymers as poly(ethylene glycol), poly(vinyl alcohol), poly(vinyl pyrrolidone) etc..[4]

Currently, RM focuses on regeneration of pathologically altered tissue architectures by using supporting scaffolds containing bioactive molecules. Scaffolds have been used for the production of different tissues and as a tool for controlled and targeted release of healing substance, growth factor, genes, and cells.[1]

2.2 Collagen as a Biomaterial

Collagen is an important biopolymer used in TE because of its self-assembling fibril forming properties, biocompatibility, low antigenicity, biodegradability and cross-linking ability, which help controlling its mechanical and water-uptake properties and degradation rate [2]. Collagen forms nearly 33 percent of all vertebrate body proteins [5].

The structure of collagen consists of three polypeptide chains forming a triple helix conformation. This is illustrated in Figure 1. Generally, two of the polypeptide chains are identical (α_1) and the third is different (α_2). Glycine is the most represented amino acid in collagen having the main function of supporting collagen triple helix by hydrogen bonding. Due to the stabilizing effect glycine makes up every third amino acid in a repeating sequence of XaaYaaGly, where XaaYaa can be any amino acid. Glycine-proline followed by any other amino acid besides the mentioned two and glycine followed by a random amino acid ending with hydroxyproline are two most frequently represented combinations of amino acid triplets in collagen. The different functional groups of the amino acids not only create hydrogen bonds between each other but also play an important role in crosslinking of collagen molecules.[5] At least 32 types of collagen have been recognized over the past years, with only three types of collagen being predominant in 80-90 percent of all tissues, which are collagen I, II and III. The structure described above depicts standardized structure of collagen type I. Complicated triple helical structure is widely represented in collagen type I (to 96 percent) but in minority represented in collagen type XII (less than 10 percent).[6][7] To this day, collagen type I is the most used collagen in TE applications, because of its structural integrity and abundance in the tissue, that is mostly treated by TE - bone, skin, tendons, ligaments and cornea [8]. The formula of collagen type I can be seen in Figure 2.

The biomedical potential of natural collagen is limited by its poor mechanical strength, thermal stability, and resistance to enzymatic degradation. Crosslinking is used for increasing the resistance of collagen-based material to enzymatic degradation and for prolonging the time necessary for material resorption in the wound site.[9] Collagen is degradable via enzymes called collagenases, also known as Zn^{2+} matrix metalloproteinases (MMPs). Collagen types I–III are hydrolyzed by MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14. The rate of the biodegradation can be further modulated by crosslinking techniques.[10] In addition, the degradation products have been shown to induce a chemotactic attraction of human fibroblasts [8]. By forming novel intra- and intermolecular bonds, crosslinking can improve collagen resistance toward mechanical stress in physiological conditions. As a biopolymer, collagen is obtained by extraction processes using acids, neutral salts or proteolytic solutions. Acid extraction with slight pepsin solubilization has proven to be the most effective technique in terms of yields. The process of extraction is destructive toward natural crosslinks of collagen and weakens the collagen structure. Thus post-processing crosslinking is needed to fabricate stable biomaterial.[8] Disadvantage of crosslinking is shown relatively decrease in cellular proliferation resulting in a lower rate of neovascularization[11]. Natural healing can take longer time and may result in permanent scars[12].

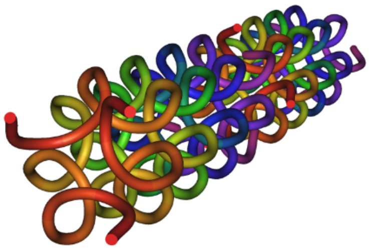

Figure 1 - Crystal structure of the collagen triple helix [13]

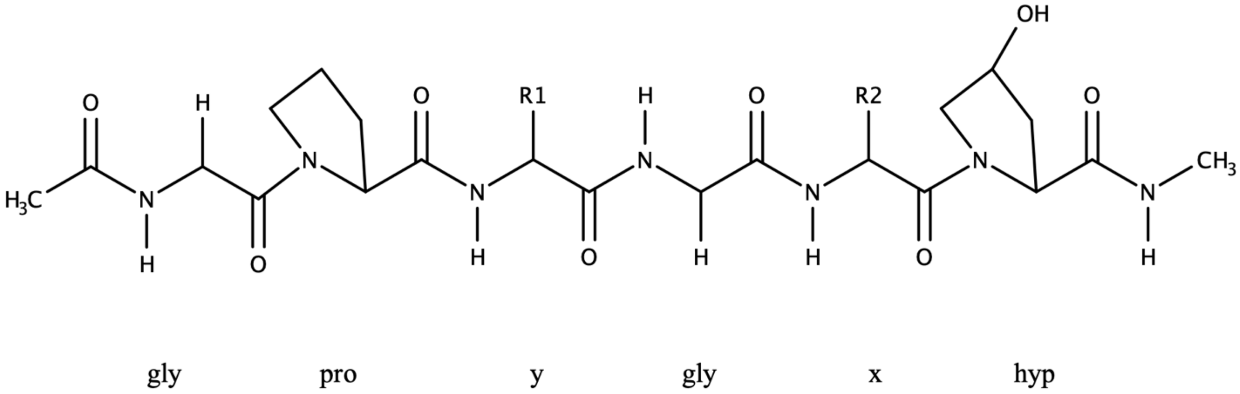

Figure 2 - Formula of collagen type I. [14]

2.3 Collagen Structure Stabilization

In the physiological environment, biopolymers often undergo mechanical stress. Thus, it is advised to stabilize them. Various exogenous chemical, physical or biological crosslinking methods are used to stabilize biopolymers. The principle lies in modification and alteration of the molecular structure by forming novel inter- and intramolecular bonds. The key idea of crosslinking process is schematically illustrated in Figure 3. Crosslinking bind single molecules into molecular net, thus preventing the molecules from sliding past each other. The overall result of crosslinking is an improvement of biomechanical properties and decrease of the degradation rate [15]. Generally, in collagen, the crosslinking agents strengthens the intermolecular forces between the triple helical structures of collagen molecules, which restricts the degree of freedom of these structures. This prevents it from unraveling the microfibrillar arrangement of collagen when the heat is added. Intermolecular crosslinks are also used for stabilizing and building resistance of collagen against enzymatic degradation. Despite all the progress on the field of crosslinked collagen, there is no standardized method for producing strong and biocompatible crosslinked collagen matrices.[9]

Moreover, by creating novel bonds and fabricating more compact structure, by method of crosslinking, the pore sizes and porosity can be changed as well [11]. This could be both advantageous and disadvantageous depending on the cell sizes. In wound healing inconveniently small pore sizes may result in scar formation.[9] The crosslinks are made between two functional groups of the polymer chains either by covalent or non-covalent bonding. This bond should reflect in some essential characteristics and properties in the scaffold.

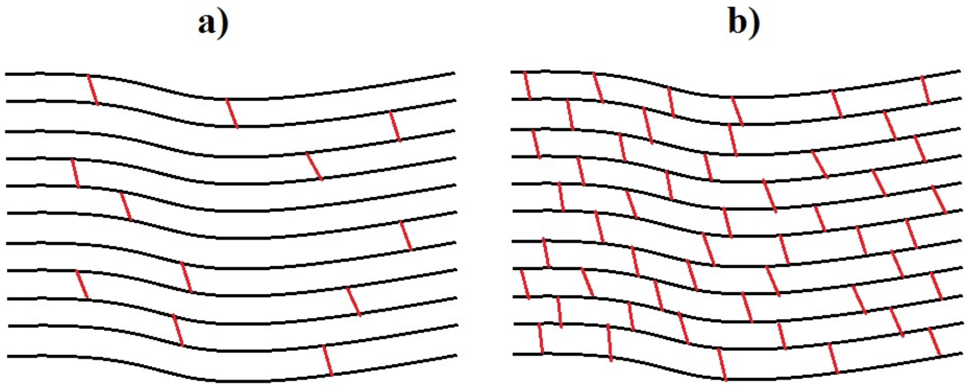

Figure 3 - Schematic illustration of newly formed covalent bonds between collagen fibers a) less crosslinking (weaker) b) more crosslinking (stronger) [16]

The ideal crosslinker should be non-toxic and completely biocompatible and should improve the mechanical features of the scaffold. The crosslinking agent depends on the chemical and biological properties of the polymer. Generally, crosslinking methodologies are divided into two categories - chemical and physical ones.[17]

2.3.1 Physical Crosslinking

Physical crosslinking has been a very popular technique of crosslinking due to the diverse application, ability to reduce toxicity and increase biocompatibility. A significant disadvantage of physical crosslinking would be in high levels of denaturation of produced collagen materials, which may influence biological properties of collagen. However, this method is a non-toxic form of crosslinking, which would be an advantage. Physical crosslinking is capable of complete sterilization of the final scaffolds, which is convenient in commercial scaffold manufacturing. Despite the fact that it is cheaper than chemical crosslinking, it loses some of its potentials because it forms far weaker bonds than chemical one.[18] The two most common ways of physical crosslinking are following:

- *Dehydrothermal treatment (DHT).* This method is based on a process where the materials are crosslinked by exposing them to heat under vacuum. Because of the high temperature, the water evaporates from the system which causes the formation of intermolecular crosslinks by the production of amide and ester bonds. This process causes the amine and carboxyl groups of the protein to bond with each other when they are in a close presence.[19] A big advantage of this method is that the material is sterilized and crosslinked repeatedly, which results in high cellular activity and immunogenic response. Studies have shown that the substance obtained from collagen and hydroxyapatite via DHT is a highly biomimetic composite for bone defect treatment.[20] Other studies were comparing DHT in two different conditions at 105 °C for 24 hours and 150 °C for 48 hours. The result pointed out that DHT at 150 °C for 48 hours improved the mechanical and biological properties of the obtained collagen scaffold, with increased pore size, permeability rate and cellular metabolic activity.[21] The *in vivo* study showed that collagen-octa calcium phosphate composite material influenced the bone tissue regeneration with the scaffold showing high resistance to enzymatic degradation [22].
- *Ultra-violet treatment (UVT).* This irradiation-based method is a simple and non-toxic method of crosslinking, which results in the linkage of the collagen. This treatment forms bonds between the amino acids with aromatic residues such as tyrosine and phenylalanine. Usually during UVT, the polypeptide chain forms bonds between each other without utilizing the acidic and basic side chains, which are essential cell recognition sites. Also, the result of this process is a highly sterile product as the process provides strong sterilization by the 254 nm ultra-violet light that can collapse the nuclei acid integrity of the microorganisms. During the process of UVT, there are two characteristic reactions which are the crosslinking and UV-induced denaturation. The presence of these two phenomena determines the mechanical properties and the degradation behavior of the product.[23] A lot of studies showed that UVT can improve the properties of the biomaterial such as rigidity, intrinsic osteogenicity and can provide an osteoconductive environment for the proliferation of the cell [24].

A representative example of crosslinking of collagen with dehydrothermal methods and crosslinking using ultraviolet radiation can be seen in Figure 4.

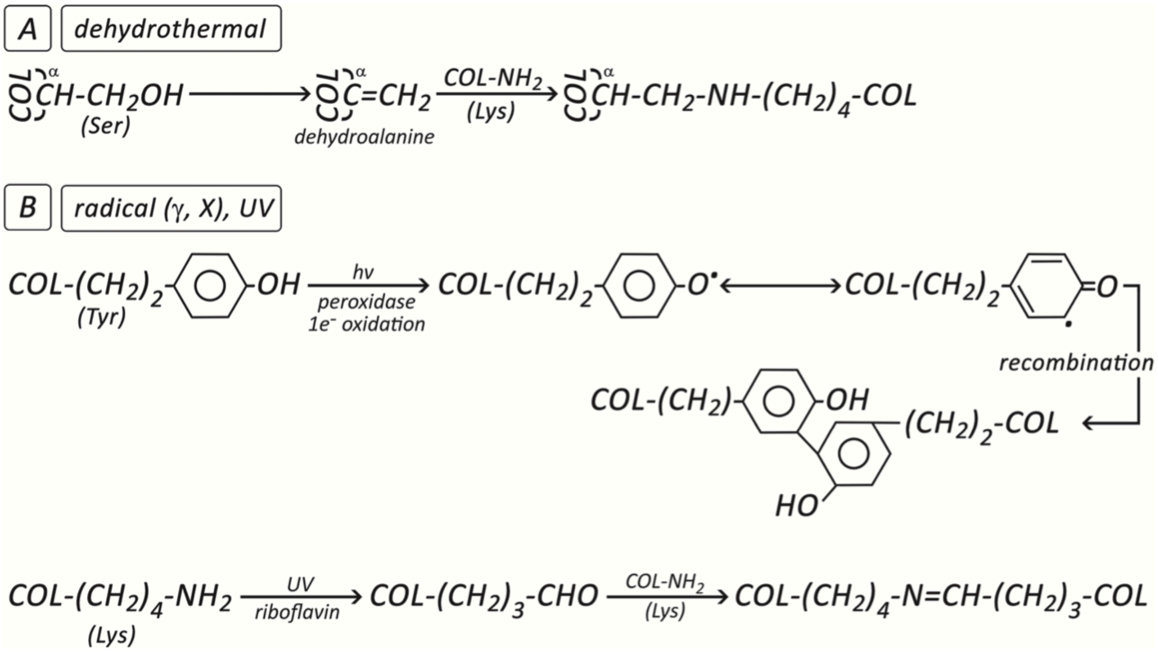

Figure 4 - Physical crosslinking of collagen: A) dehydrothermal crosslinking and B) crosslinking using radical oxidation and UV light [25]

2.3.2 Chemical Crosslinking

Chemical crosslinking is mainly used in RM, because of its rapid ability to undergo chemical modification which results in a higher crosslinking degree. The main advantage of chemical crosslinking would be its capability of not changing the chemical structure of the used biopolymers. It forms far stronger bonds than physical crosslinking, which can stabilize the proteins and other biopolymers very well. However, this method is often toxic for the cells and requires a washing procedure of the scaffolds to remove the residual crosslinker. Due to this fact it is also more expensive than physical crosslinking. Because of its cytotoxicity, it is required to find non-toxic solutions for chemical crosslinking. The most commonly used crosslinking agents are glutaraldehyde, carbodiimide agents and epoxy compounds.[23] There is also a wide spectrum of potential non-toxic crosslinkers, for example, lysyl oxidase, genipin, citric acid and so on [18].

- *Glutaraldehyde (GA).* GA as a chemical crosslinker is used to stabilize various types of biopolymeric scaffolds, hydrogels, and composites. It can radically improve the mechanical properties and durability of the biomaterials in the physiological environment.[24][26] GA reacts with the amine or hydroxyl groups of the proteins through a Schiff-base reaction using intra- or intermolecular forces to form bonds between the polymeric chains. Every available amine group that is present in the biopolymer reacts with GA and forms a strong crosslinked product.[27] Therefore, GA crosslinking method has been the golden standard in RM and TE. Scaffolds crosslinked with GA are used for transplantation procedures, such as heart valves replacement.[28] These scaffolds show a significant increase in tensile strength and are antigenic [29]. However, the aldehyde group of GA is highly cytotoxic and cause inflammation in the human organism [30]. There are many detoxifying methods to make the scaffolds more compatible with the body. Washing the crosslinked scaffolds out with solutions that contain amine groups increase the biocompatibility of the scaffolds by inducing reaction between amine and aldehyde groups.[31] Even though GA can cause cell toxicity it is a widely used crosslinker. The fact that it has biohazard problems make its application in commercial products rather difficult.[23] The basic crosslinking scheme of collagen with GA can be seen in Figure 5.

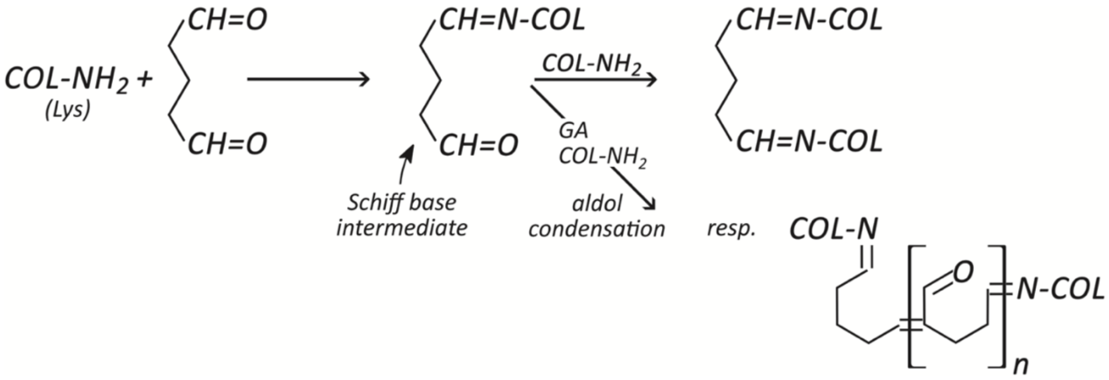

Figure 5 - Crosslinking scheme of collagen with glutaraldehyde and of following aldol condensation [25]

- *Carbodiimide crosslinkers.* These crosslinking providing chemicals have a functional group consisting of C=N=C bond. They are water-soluble compounds that can react with a lot of other functional groups, such as carboxyl, hydroxyl and sulfhydryl groups.[23] Carbodiimide crosslinkers are only active at acidic pH values.[32][33] Also, there are some reactions that take place at neutral pH values as well. A huge advantage of carbodiimide crosslinkers is that all of the residues are water soluble (e.g. urea derivatives), so they can be washed out of the final scaffold. These scaffolds are less cytotoxic than GA but show poor biochemical properties and rapid degradation.[34] The crosslinking scheme of collagen with EDC in combination with NHS as a carbodiimide crosslinker can be seen in Figure 6.

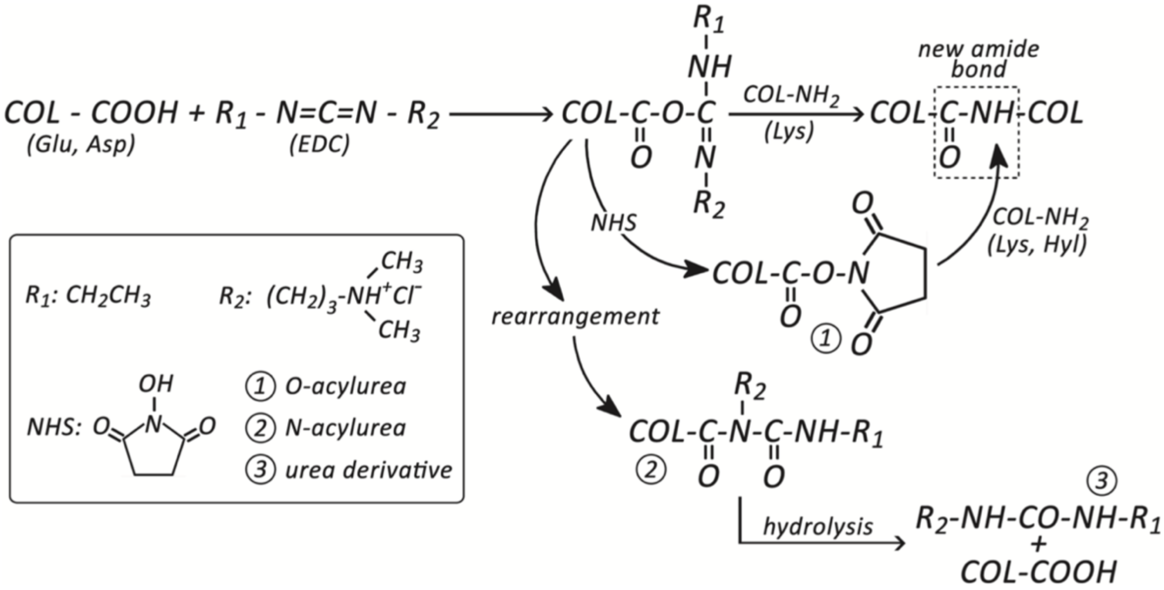

Figure 6 - The crosslinking scheme of collagen with EDC in combination with NHS [25]

- *Epoxy compounds.* These organic compounds consist of a three-membered ring with one oxygen atom and two carbon atoms. They are able to react with an amine, carboxyl, and hydroxyl groups. The crosslinking scheme of the way collagen reacts with the epoxy group can be seen in Figure 7. They are mainly used in bone tissue engineering.[35][36] Ethylene glycol diglycidyl ether is an epoxy-based material that is commercially used in the chemical industry. It has a linear structure with bulky side chains and the use of so-crosslinked material is limited bone tissue engineering. The cytotoxicity of these compounds is almost equal to the GA scaffolds. Crosslinked materials are highly resistant to enzymatic degradation.[37][38]

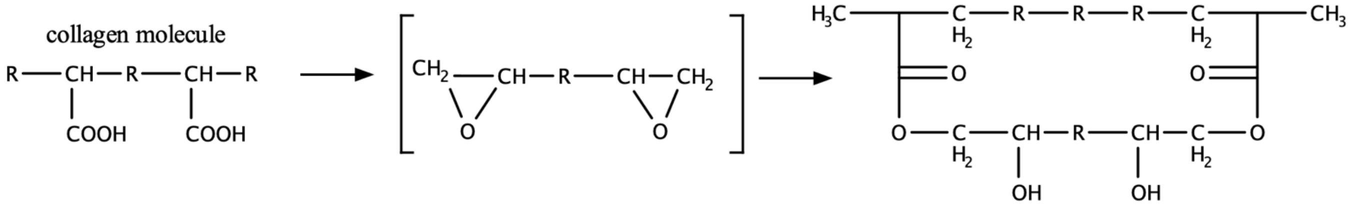

Figure 7 – Scheme of the way which the epoxy group reacts with carboxy groups of collagen [39]

2.4 Novel Crosslinking Alternatives

There are a lot of different methods of physical, chemical or biological crosslinking to produce crosslinked collagen scaffolds, but nearly every method has its physiological and mechanical drawbacks. Chemical crosslinking agents are usually cytotoxic and often toxic solvents have to be used to dissolve the agents in order to be functional. Hence there are limitations in using these crosslinking agents in RM.[38] The main focus of various studies is to find a natural crosslinking agent that would propose a solution to these problems because of its high biocompatibility. These agents should be further optimized, and their performance parameters should be evaluated. Novel crosslinking alternatives that should be considered are discussed below.[18]

- *Lysyl oxidase (LO)*. Protein lysine 6-oxidase is a copper-containing quinoprotein with a lysyl adduct of tyrosyl quinone at its active center. It is used to produce LO, which is an enzyme that can be used as a crosslinker agent.[40] The copper in LO has a purpose of transferring electrons to produce oxygen to support the oxidative deamination of peptidyl lysyl groups situated in collagen. By oxidizing the peptidyl lysine, peptidyl α-aminoadipic-δ-semialdehyde is produced.[41] This product has the ability to spontaneously react with other aldehyde or lysyl groups. This way it can be used to form intra- and interchain crosslinks.[42] Except for the primary structure of LO, there are also binding domains for cytokines, for example, growth factor β and cell-surface adhesion proteins [43]. LO is most cooperative with collagen and elastin, but it can crosslink simple monoamines, diamines, and histones [44]. The partial reactions of crosslinking by lysyl oxidase are illustrated in Figure 8.

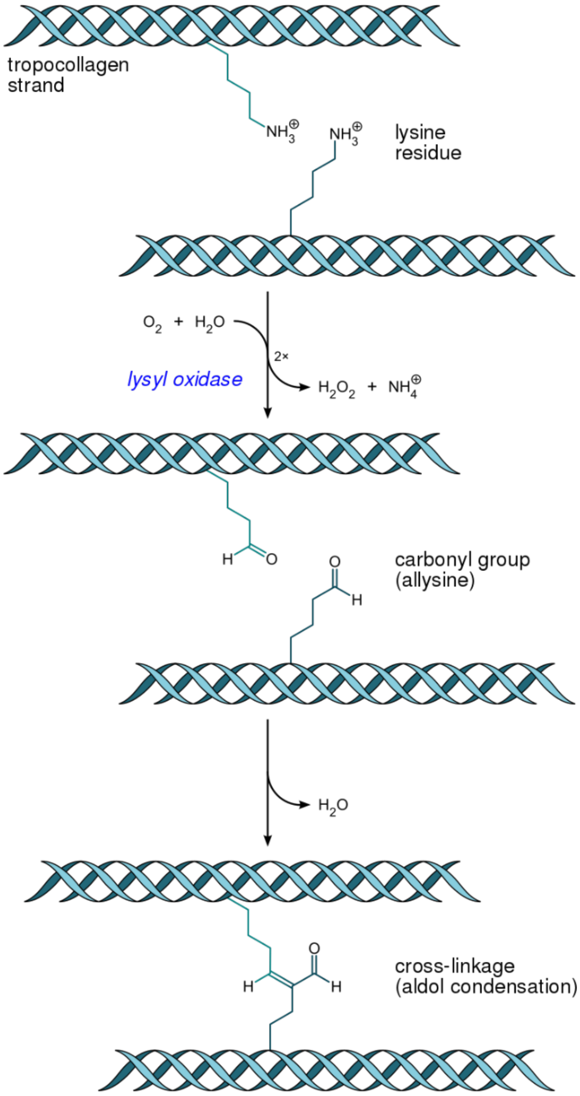

Figure 8 - Crosslinkage of tropocollagen strands through aldol condensation of allysine resins after oxidation of lysyn resins by lysyl oxidase [45]

- *Genipin (GE).* GE has recently become a very popular crosslinking agent because it is a natural compound with high biocompatibility. GE is contained form a fruit called *Gardenia jasminoides Ellis.* It is the hydrolytic product of geniposide. It has a lot of active groups and can react with amino acids forming blue pigments. Studies showed that GE is about ten thousand times less cytotoxic than GA.[24] GE is mainly used for collagen, gelatin, and chitosan crosslinking. It can produce stable and biocompatible scaffolds [46][47]. GE stimulates the crosslinking of free amino groups, together with lysine, hydroxylysine, and arginine [48]. During the crosslinking of collagen, the reaction is mediated by cyclic groups. The scaffolds produced with GE show enhanced biocompatibility to those produced with GA.[49] To produce GE scientists crush the mentioned fruit and they extract it with chloroform and methanol. After the product is dried and recrystallized using acetone. This procedure produces the geniposide crystals which ate further mixed with an aqueous solution of β-glucosidase with acetate buffer. After the system was extracted again with ether and crystalized forming colorless GE crystals. GE is used to produce scaffolds which have high microcapsule strength and permeability characteristics. However, this method of crosslinking depends on pH and the concentration of GE. With higher GE concentration the scaffolds release the encapsulated substances slower. Also, GE crosslinked scaffolds demonstrate longer degradation periods, higher antibiotic elution, enhanced thermal stability, and mechanical properties. Owing to its biocompatibility biodegradability and mono-dispersibility makes GE a potential candidate in several biomedical applications.[50] Two mechanism schemes of crosslinking of collagen with genipin can be seen in Figure 9.

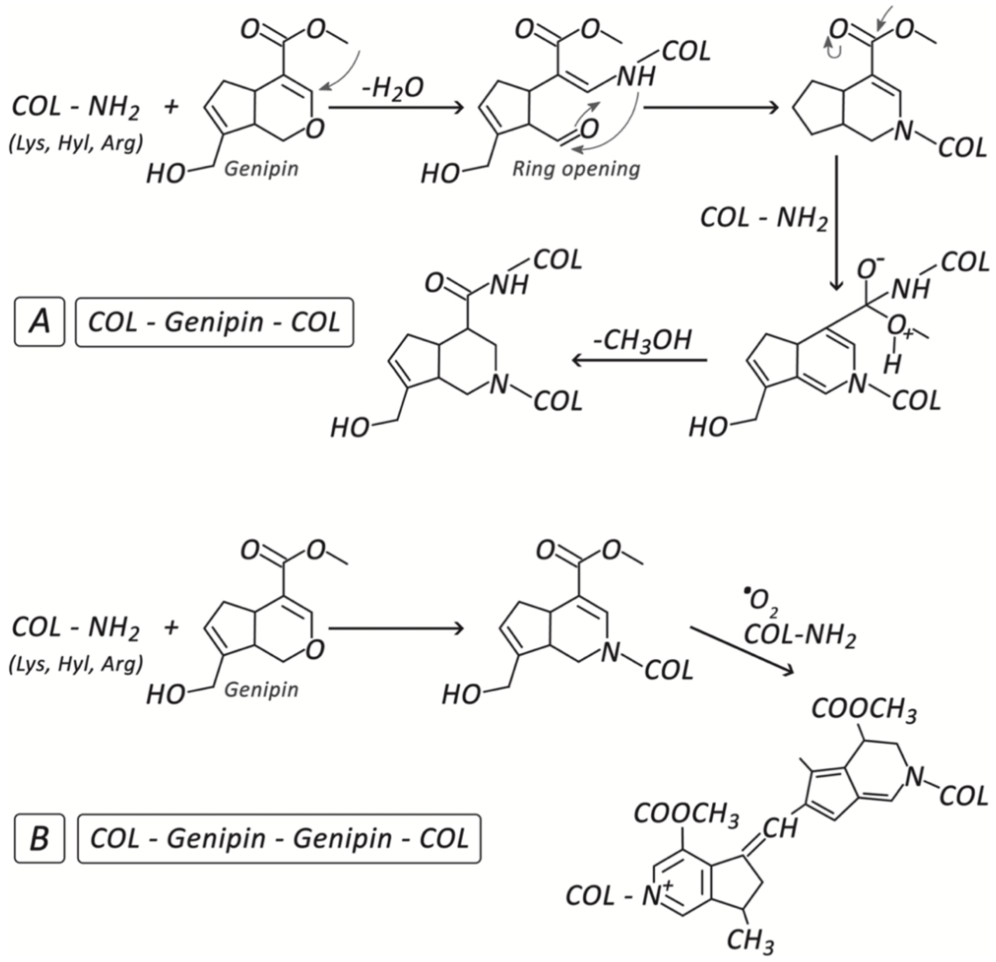

Figure 9 – Two mechanisms of crosslinking of collagen with genipin; scheme A results in one genipin molecule between two collagen reaction sites, while scheme B results in dimerization of genipin molecule between two collagen reaction sites [25]

- *Citric acid (CA).* This weak organic acid that can be found naturally in citrus fruits and is widely used in cosmetics, pharmaceuticals, foods, and drinks has been recently fount to provide crosslinking abilities [51]. CA is able to react with hydroxyl and amine groups. It is also able to crosslink proteins and polysaccharides [52][53]. During the crosslinking with CA, the system forms ester bonds, which increases hemocompatibility [24]. CA is a non-toxic crosslinker that contains three carboxylic functional groups that can form stable amide bonds with collagen. One of its advantages is that the reaction can be approached by different pathways. These different pathways depend on different pH values and its effect on ionization, temperature and the presence of a catalytic agent. Scaffolds crosslinked with CA are mainly produced by electrospinning. During this process, the CA solution pH values should be approximately 3,5 and heat treated for at least 16 hours at high temperatures. The scaffolds produced by this method show the highest rate of crosslinking between the CA and collagen.[54] Crosslinking with CA can increase hemocompatibility, balance the hydrophilicity of the polymers provide binding sites for bioconjugation [24]. Due to its low cytotoxicity, low price and availability CA should be considered as an alternative crosslinker [52]. A basic scheme of crosslinking of collagen with citric acid derivative (CAD) can be seen in Figure 10.

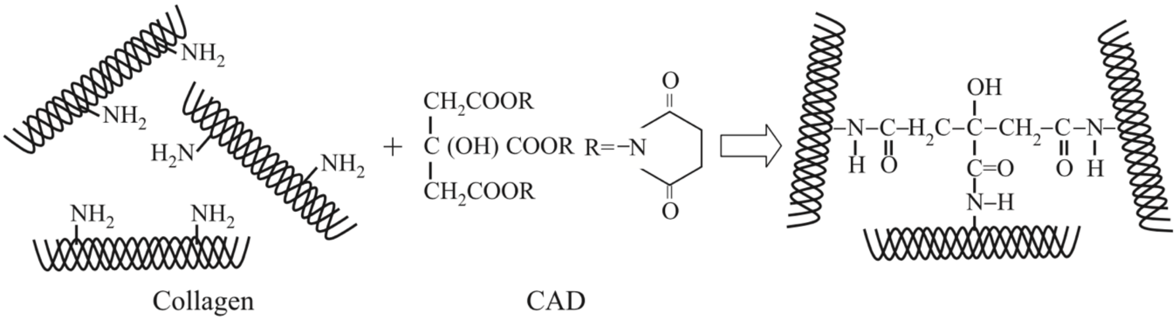

Figure 10 - Crosslinking of collagen with CAD [55]

- *L-Arginine (LA).* LA is an α-amino acid. It contains α -amino group, an α-carboxylic acid group, and a side chain consisting of a 3-carbon aliphatic straight chain ending in a guanidine group. LA is classified as an essential amino acid therefore, it is highly biocompatible and can be considered as a non-toxic crosslinking agent. The use of this amino acid as a crosslinker adds structural stability to the produced scaffolds. LA improved the porous architecture of the scaffolds and enhanced their swelling ability and biostability. The crosslinking scheme of collagen with LA can be seen in Figure 11. It also plays an important role in defining the scaffold microstructure as well as guiding the cells on the matrix surface. LA crosslinked scaffolds show faster-wound healing as the non-crosslinked ones. It is a very promising alternative for RM and TE because of the excellent re-epithelization and dense collagen fiber deposition capabilities of these scaffolds.[52]

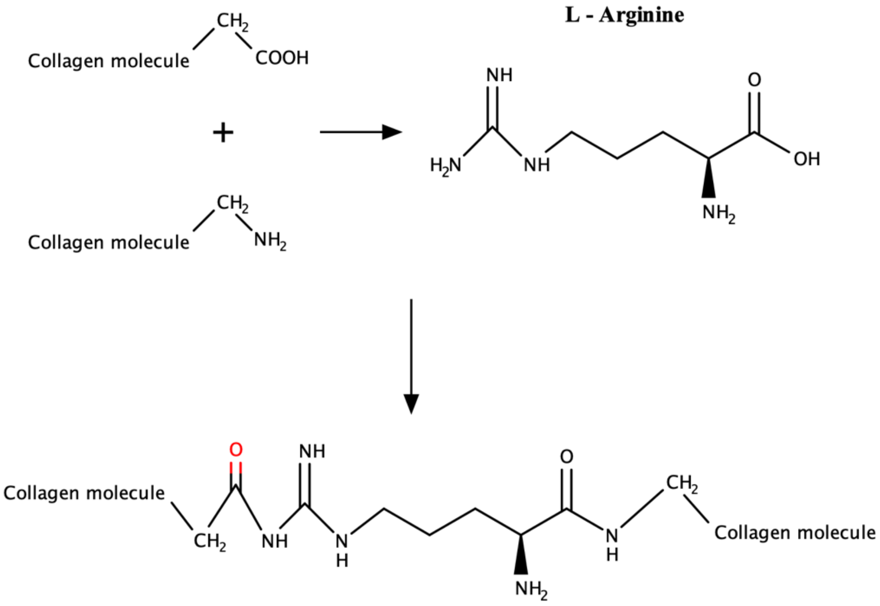

Figure 11 - The crosslinking scheme of collagen with L-Arginine [56]

- *Tannic acid (TA).* Hydrolysable tannins can be used to crosslink collagen fibrils using hydrogen bonding and hydrophobic effects. It has been studied because of its practical application in wound healing and TE. It can be used not only just for crosslinking, but it has a capability to lower the chance of breast cancer. TA-crosslinked collagen scaffolds can be used to target the disruption of cancerous cell proliferation while they have no effect on normal human body cells.[57] Even though TA is very effective, it produces a lot of residues which, requires a washing process after the crosslinking to wash out the remaining residues [58]. TA crosslinking induces collagen fibril merging resulting in a decrease in pore size of collagen scaffolds. It also increases the strength and elastic modulus of collagen gels. TA shows considerable promise to be incorporated as an anti-carcinogenic agent and crosslinker in RM and TE.[59] A scheme of crosslinking of collagen with TA can be seen in Figure 12.

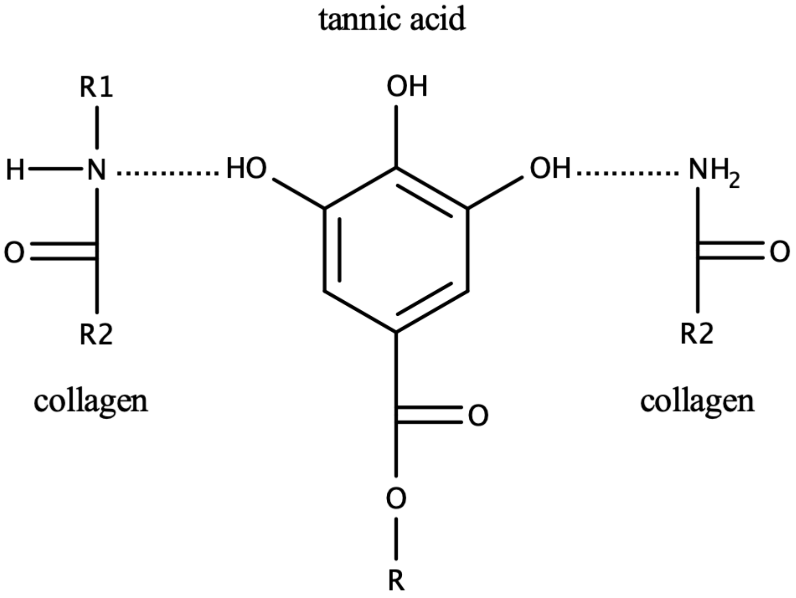

Figure 12 - Hydrogen bonds in the crosslinking of collagen with TA [60]

- *Polydopamine (PDA).* The collagen matrix is modified via dopamine self-polymerization and then collagen immobilization. This way the biological, mechanical and physicochemical properties of the collagen scaffolds were enhanced. The polymerization of dopamine can be regulated during the process by controlling the number of hydrogen bonds formed between the phenol hydroxyl group and nitrogen atom within the collagen fibers. The collagen scaffolds coated by PDA show better mechanical properties, thermal stability, and surface hydrophilicity. Scaffolds crosslinked with PDA are highly biocompatible and have stronger cellular attachment than non-crosslinked scaffolds. PDA used in coating crosslinking method produces a porcine acellular dermal matrix with excellent biological and mechanical properties. This crosslinking method provides scaffolds with outstanding cellular performance and minor toxicity with very little side effects. Therefore, this alternative may be an appropriate platform for TE and RM.[61] A schematic representation of new bonds between collagen and PDA during crosslinking can be seen in Figure 13.

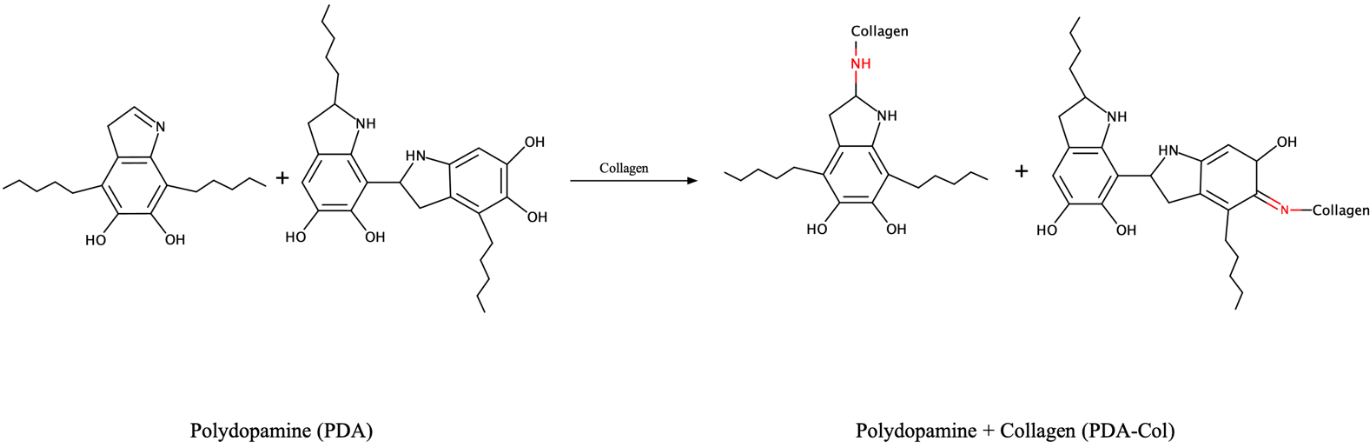

Figure 13 - Crosslinking of collagen with PDA [62]

2.5 Riboflavin

Riboflavin (RF) was discovered by Blyth in 1872, but it was first proclaimed as a vitamin in the early 1930s. It was discovered as a yellow fluorescent pigment in milk. When it was first isolated from milk, although not purified, the compound was given the name lactochrome.[63][64] Riboflavin is essential for antioxidant protection and also for nutrient metabolism [65]. From the chemical point of view riboflavin is known as vitamin B_2. It is a water-soluble vitamin with the systematic name 7,8-dimethyl-10-ribityl-isoalloxzine. The chemical structure of riboflavin can be seen in Figure 14. The two most important forms of riboflavin are flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These compounds can be found in a wide range of redox reactions, some of which are essential to the function of aerobic cells. Vitamin B_2 and its derivatives are present in a wide variety of groceries. The most important product containing the highest amount of riboflavin is milk and its products. Therefore, riboflavin deficiency is present in populations consuming little milk or milk products. Some scientist claim that riboflavin deficiency may contribute on cardiovascular diseases, some types of cancer and is associated with weakened handling of iron and night blindness.[66] Wide variety of animal, plants and microorganisms are able to produce riboflavin, but because of its absolute necessity for the human health it should be provided by the diet.[64] During riboflavin deficiency a higher amount of riboflavin intake is appropriate. There are no records of toxic effects when higher riboflavin doses are ingested by a human due to its solubility in body liquids and its excretion in the urine, however it can be suggested that an excessive dose of riboflavin could cause an imbalance in the antioxidant state of human body.[63][65]

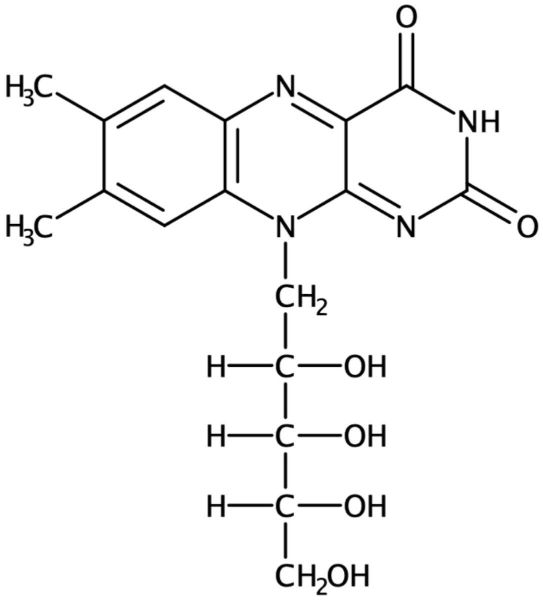

Figure 14 - Chemical structure of riboflavin [67]

After chemical processing it forms orange-yellow crystals and in its pure form it is slightly soluble in water. One gram dissolves in 3-15 liters of water, depending on the structure of the crystals.[66]

2.5.1 Absorption and Fluorescence Spectra of Riboflavin

Calculating accurate and precise spectra of riboflavin has proven very difficult for computational chemistry. Time-dependent density functional theory has been used to determine ultraviolet and visible spectra of riboflavin. However, the calculated spectra often deviated from experimental results when calculating the spectra in vacuum. While dry riboflavin is stable, in alkaline solution it degrades rapidly. The absorption maxima of riboflavin in the human body are in the interval of 445 - 450 nm. As a consequence, blue visible light causes degradation of riboflavin. Most of the predicted spectra determined by various methodologies were shifted compared to the experimental data. The alterations were in between 20 and 40 nm.[68] However, the fluorescent absorption and emission spectra of riboflavin show a spectrum with a lower peak at 374 nm, and a higher peak at 445 nm, while the maximum emission wavelength was at 524 nm [69]. The fluorescent absorption spectra of riboflavin are showed in Figure 15.

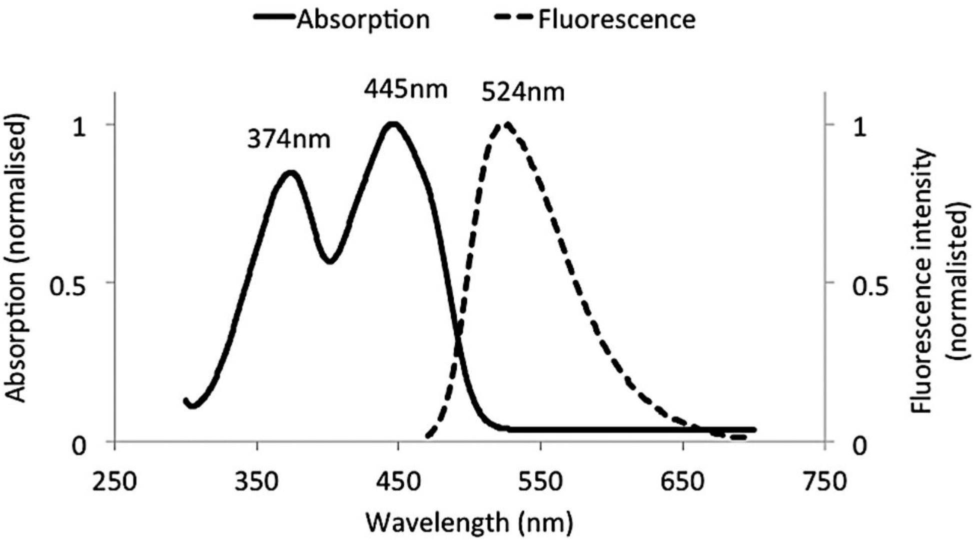

Figure 15 - The absorption and fluorescence emission spectra of riboflavin in saline [69]

2.5.2 Collagen Crosslinking Mediated by Riboflavin and UV-light

The ability of collagen to be crosslinked with riboflavin in combination with ultraviolet light is used in various treatments. One of them is the treatment of keratoconus, which is a corneal degeneration often described as bilateral conical protrusion and corneal thinning. The severity of the illness has different stages. It can appear as slight irregular astigmatism to serious visual impairment.[70] The cause of this disease is unknown, however, it is known that the biomechanical characteristics of the cornea result from the collagen scaffold and collagen compound and their bonding with the collagen fibrils. Precisely this 3-dimensional configuration of the collagen lamella establishes the resistance of the cornea.[71] The method of treating this condition using photooxidative crosslinking of collagen with the use of riboflavin and ultraviolet light. This allows an extra covalent binding between the molecules of the collagen resulting in stabilization of collagen fibers.[72] The riboflavin and the ultraviolet light add stiffness and resistance against proteolytic enzymes to the cornea [73].

The crosslinking is achieved thanks to the two important functions of riboflavin. Vitamin B_2 is capable of absorbing ultraviolet irradiation, due to this fact it acts as a photosensitizer for the formation of reactive singlet oxygen. As a result of ultraviolet light irradiation riboflavin forms radicals that are responsible for the crosslinking. During crosslinking, 370 nm wavelength is used, which is riboflavin’s absorption maximum. Thanks to this, riboflavin absorbs 95 % of ultraviolet light and after sufficiently penetrating the stroma it forms a shield for the deeper tissues of the eye-protecting it from further damage.[74] The ultraviolet light is effective only where it is absorbed, but this absorption riboflavin acts as a photo-mediator. It synthesizes free radicals to induce the formation of new covalent bonds between the collagen fibers. After irradiation of riboflavin and the formation of free oxygen radicals, these radicals induce the change of amino groups at the end of each collagen string to other groups, often aldehyde groups. Afterward, these reactive groups can form new covalent bonds. This process is schematically illustrated in Figure 16. This happens in an intermolecular manner where the structure of collagen is strengthened by the formation of covalent bonds between its fibers producing more bonds than before. The product of this reaction has better physical properties, such as higher stability and a more compact structure. [75] Maybe the biggest advantage of this crosslinking method is the ideal properties of riboflavin. Because of its biocompatibility and essentiality to the human body, it does not show any kind of cytotoxicity compared to conventionally utilized photo-initiators. It improves various mechanical properties and delays enzyme-triggered degradation of collagen scaffolds. This method is used for numerous treatments, not only for treating eye damage like keratoconus, but also some types of knee injuries, such as torn meniscus. In recent years, collagen-based scaffolds have been used in meniscus tissue engineering.[76]

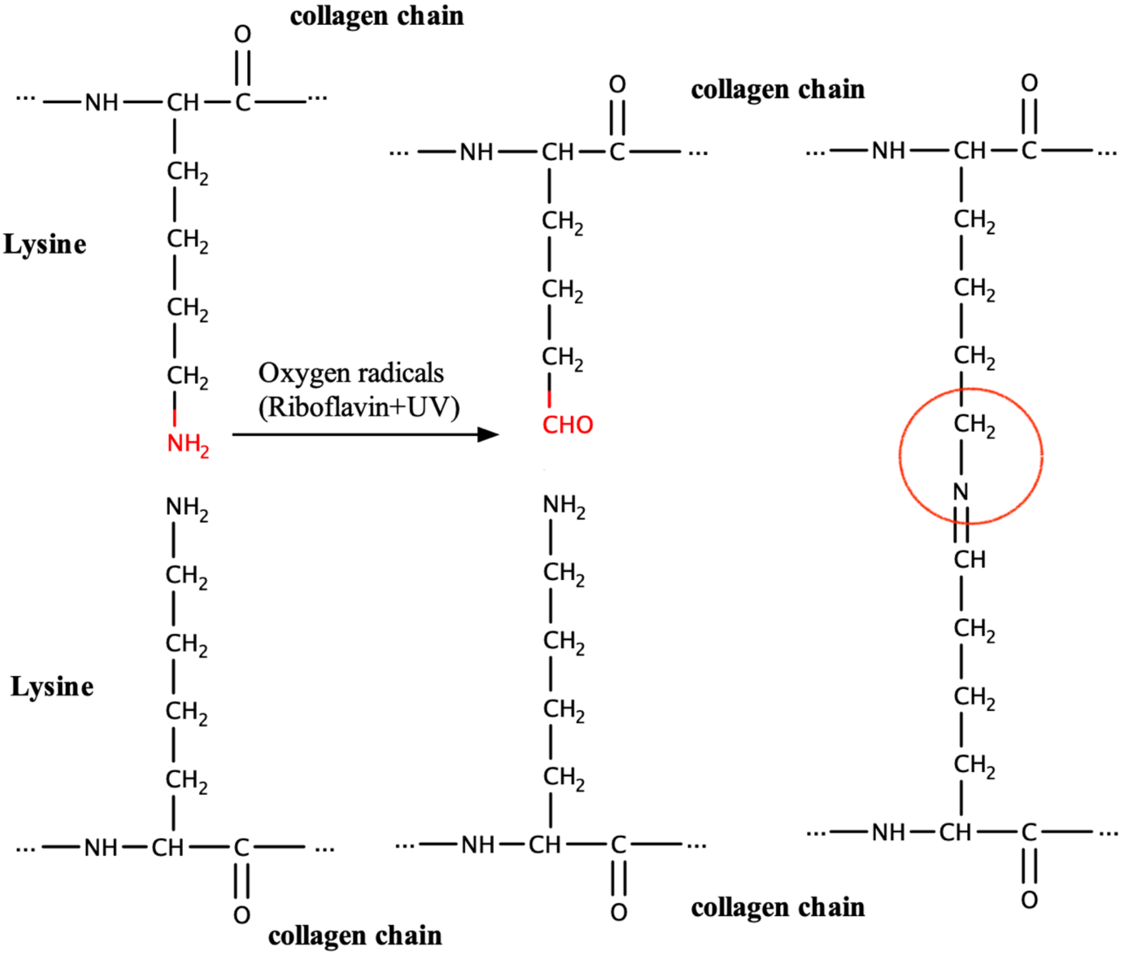

Figure 16. Schematic illustration of collagen crosslinking with riboflavin and ultraviolet light [75]

3 GOAL OF THE WORK

The goal of this work is to evaluate efficiency of photo-crosslinking method for porous collagenous scaffolds using riboflavin as an initiator irradiated by both shortwave (UV-C) and longwave (UV-A) light. Photo-stabilized scaffolds will be physico-chemically characterized gaining a complex knowledge about the impact of the crosslinking on stability, morphology, porosity and chemical structure of the collagenous scaffolds.

The steps of this work include:

- Preparation of collagenous scaffolds
- Chemical crosslinking using EDC/NHS
- Photo-crosslinking in aqueous and ethanolic solutions of RF using UV-C and UV-A as a mediator
- Study of morphology and porosity using confocal laser scanning microscopy (CLSM)
- Study of collagen structure and triple helical structure integrity using Fourier-transformed infrared spectroscopy (FTIR)
- Study of stability and the swelling of collagenous scaffolds in a physiological solution with Ca^{2+}, and swelling ratio calculation
- Study of physiological degradation in a physiological solution with Ca^{2+}
- Evaluation of the results and effect of different RF concentrations and UV irradiation time on the stability of the prepared collagenous scaffolds

4 EXPERIMENTAL PART

4.1 Chemicals

- Bovine collagen COLADO 100% lyophilisate.
- Ultra-pure water type II according to ISO 3696.
- Ethanol 96% g.r., PENTA, Czech Republic.
- Riboflavin-5'-phosphate sodium salt dihydrate, Alfa Aesar™, USA.
- N-(-3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Sigma Aldrich, Germany (EDC).
- N-hydroxysuccinimide, Sigma Aldrich, Germany (NHS).
- Disodium hydrogen phosphate dodecahydrate G.R., Lach-ner, Czech Republic.
- Physiological solution with Ca^{2+} ions (Solution A) made from:
    - Sodium chloride G.R., Lach-ner, Czech Republic.
    - Calcium chloride dihydrate G.R., Lach-ner, Czech Republic.
- Collagenase from Clostridium histolyticum, Sigma Aldrich, Germany.

4.2 Instrumentation

- Disintegrator (IKA – T18 digital – ULTRA TURRAX).
- UV lamp (type M101HO, 11 W, UV Tools, USA).
- UV fluorescent tube for longwave UV-A, 365 nm, 6 W, Phillips, Netherlands.
- UV fluorescent tube for shortwave UV-C, 250 nm, 6 W, Phillips, Netherlands.
- Heating-block thermostat, model TK23, DITABIS, Germany.
- Analytic scales, model AV264C, OHAUS, USA.
- Magnetic stirrer, type RCT B S000, IKA, Germany.
- Freeze dryer, type EPSILON 2-10, Martin Christ, Germany.
- Confocal laser scanning microscope, type LEXT OLS4100, OLYMPUS, Japan.
- Fourier-transformed infrared spectroscope HYPERION 3000/Vertex 70V, Bruker.
- Incubator, Thermo-Star-Desiccator, SICCO, Germany.

4.3 Softwares

- ImageJ, National Institutes of Health (NIH), Bethesda, Maryland, U.S.
- Microsoft Excel, Microsoft Corporation, Redmond, Washington, U.S.
- OPUS software, Bruker Corporation, Massachusetts, U.S.
- Origin8, OriginLab Corporation, Northampton, Massachusetts, U.S.

4.4 Fabrication and stabilization of porous collagen scaffolds

4.4.1 Preparation and freeze-drying of collagen suspensions

In the preparation of 0,5% collagen solution, collagen scaffolds were made by weighing previously calculated amount of 100% freeze-dried bovine collagen into ultra-pure water and left it swell in the fridge for 30 minutes. The homogenous collagen suspension was prepared by disintegrating the collagen fibers using IKA disintegrator. During the disintegration the solution was kept cooled down in water bath to prevent unwanted degradation of collagen. Afterwards, the collagen suspension was pipetted into 48 wells cell culture plates used as a mold. The suspension intended to be crosslinked using UV light irradiation was pipetted according to schemes in Figure 17 (a) preventing the unwanted irradiation of adjacent row.

The suspension intended to left non-crosslinked or crosslinked with chemical crosslinkers were pipetted according to scheme in Figure 17 (b). Afterwards collagen suspensions were lyophilized for 48 hours to form porous collagen scaffolds.

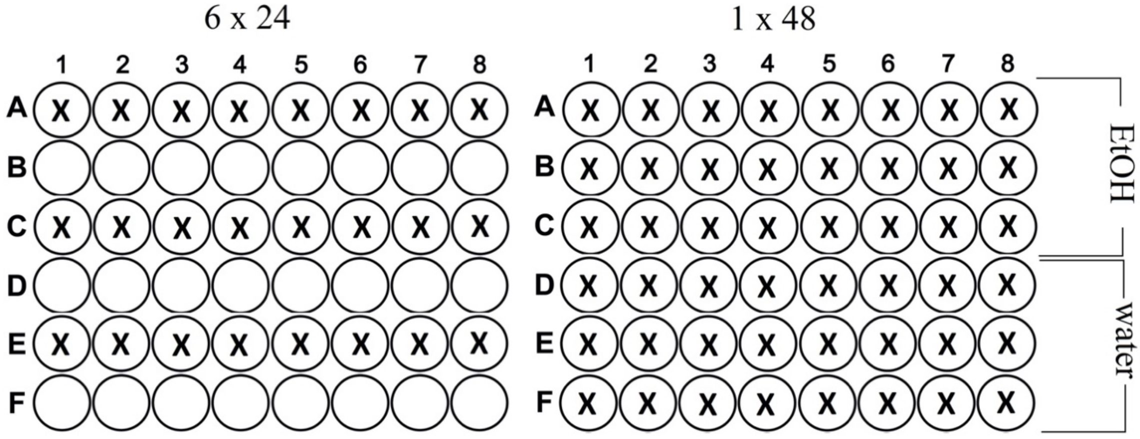

Figure 17 - Pipetting scheme of collagen suspensions. Left (a), right (b)

4.4.2 Chemical crosslinking using EDC/NHS

The prepared and lyophilized collagen scaffolds were soaked in a crosslinking ethanolic solution of 50 mM N-(-3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 25 mM N-hydroxy succinimide (NHS) for 2 hours according to our published optimized procedure [77]. After crosslinking, stabilized collagen scaffolds underwent a washing process including two washes of 0.1 M Na_2HPO_4 and three washes of ultrapure water with intervals of 30 minutes.

4.4.3 Photo-crosslinking using riboflavin

Two sets of riboflavin-based crosslinking agents were prepared. One aqueous and one non-aqueous solution in ethanol. General procedure of photo-crosslinking followed this procedure. Serie of collagen scaffolds were soaked in 250 µl of crosslinking aqueous solution or ethanolic solution of riboflavin. After pipetting the crosslinking agent to the scaffolds two lines of collagen fibers were shielded by thin foil while the remainder line was irradiated with a UV lamp (type M101HO, 11 W, UVTools, USA) with UV-A or UV-C fluorescent tube. After the irradiation, the process was repeated on all of the collagen scaffold lines. During irradiation with ultraviolet radiation, the scaffolds containing riboflavin showed fluorescence. This happened due to the fact the riboflavin is fluorescent when exposed to UV light. This effect can be seen in Figure 18. After the crosslinking process, the scaffolds were put in a freezer and later in a lyophilizer. The riboflavin solution was not rinsed out of the collagen scaffolds.

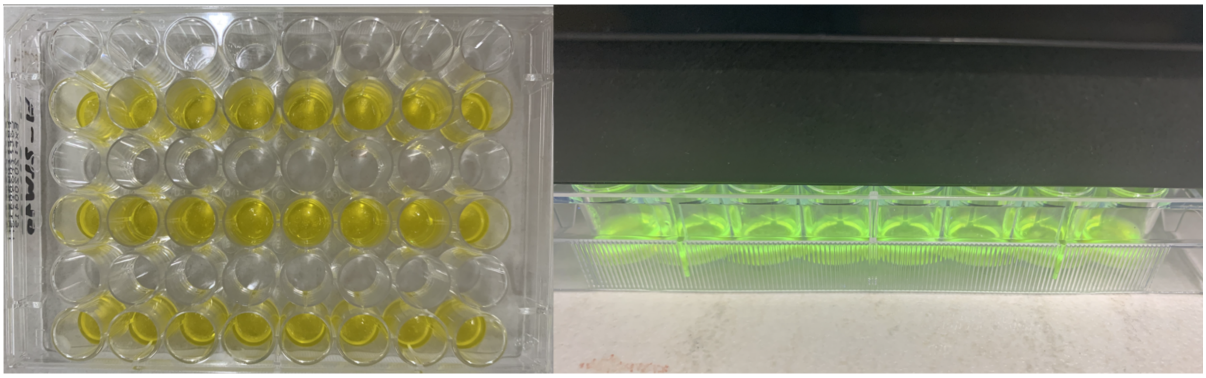

Figure 18 - Collagen scaffolds with crosslinking agent and fluorescence of riboflavin during irradiation with UV light

4.4.4 List of prepared samples

For the different studies of collagen scaffolds the samples were prepared in EDC/NHS, aqueous and alcoholic solutions of riboflavin. Pure non-crosslinked collagen was put both in water and ethanol as a reference. Shortwave and longwave UV light was used for the crosslinking of the collagen scaffolds. For different type of samples, the pair of UV lights were applied to various concentrations of riboflavin crosslinking agent and various time intervals. In Table 1 an overview of all types of prepared collagen scaffolds is depicted, the concentrations of the riboflavin solution and length of irradiation is listed also.

Table 1 - List of prepared collagen samples

Sample type	Sample name	t (UV-A) [min]	t (UV-C) [min]	c (riboflavin) [µM]	c (EDC/NHS) [mM]	Solvent
Non-crosslinked	pureColl_W	-	-	-	-	Water
	pureColl_E	-	-	-	-	Ethanol
Chemically crosslinked	crossColl	-	-	-	50 mM / 25 mM	Ethanol
Effect of solution	LWcrossW	5	-	1000	-	Water
	LWcrossE	5	-	1000	-	Ethanol
	SWcrossW	-	5	1000	-	Water
	SWcrossE	-	5	1000	-	Ethanol
Effect of irradiation time	LW5min	5	-	1000	-	Water
	LW10min	10	-	1000	-	Water
	LW15min	15	-	1000	-	Water
	LW30min	30	-	1000	-	Water
	SW5min	-	5	1000	-	Water
	SW10min	-	10	1000	-	Water
	SW15min	-	15	1000	-	Water
	SW30min	-	30	1000	-	Water
Effect of riboflavin concentrations	LW1µM	15	-	1	-	Water
	LW10µM	15	-	10	-	Water
	LW100µM	15	-	100	-	Water
	LW1000µM	15	-	1000	-	Water
	SW1µM	-	15	1	-	Water
	SW10µM	-	15	10	-	Water
	SW100µM	-	15	100	-	Water
	SW1000µM	-	15	1000	-	Water

4.5 Physicochemical characterization

4.5.1 Morphology and porosity evaluation

The influence of the selected crosslinkers (EDC/NHS, riboflavin in ethanol, riboflavin in water) on structure quality, morphology and porosity of prepared collagenous scaffolds was evaluated using confocal laser scanning microscopy (CLSM, type LEXT OLS4100, OLYMPUS, Japan). With any further preparation of the samples, lyophilized collagenous scaffolds were placed under the objective of the microscope. Images were acquired with magnification of 5x, 10x, 20x, 50x, 100x.

Porosity was evaluated using software ImageJ. 50 pores were measured in 2 directions on each type of crosslinked collagen scaffolds, the longest and the shortest. The results from the porosity analysis were processed in the software Excel.

4.5.2 Evaluation of collagen structure and triple helical structure integrity

The effect of UV irradiation in combination with riboflavin on collagen structure and integrity of collagen triple helix was observed using Fourier-transformed infrared spectroscope (FTIR, type HYPERION 3000/Vertex 70V, Bruker) with vacuum system. With any further preparation, samples were placed on diamond crystal and measured. Background signal obtained prior the measurement of the samples was automatically subtracted from samples' spectra in OPUS software. Further spectral analysis was performed in software Origin8.

4.5.3 Stability in simulated physiological environment

The stability of prepared collagenous scaffolds was evaluated gravimetrically. The stability studies were carried out according to following procedure. The porous collagen scaffolds were put in pre-weighed vials. The vials were weighed with and without the dry collagen scaffolds to acquire dry collagen scaffold weight by subtraction. The scaffold was then immersed into the Solution A, a physiological solution with Ca^{2+}, which was prepared prior to the measurement. After 1 minute the saline was sucked out of the vial using a syringe and the drenched collagen scaffold was weighted to acquire weight of swollen wet sample in the time interval of 1 minute. The saline was injected back into the vial after weighing. The sample was re-weighted during 2 hours in time intervals 3; 5; 10; 15; 30; 45; 60; 90; 120 minutes. During the whole measurement, the vials with samples were placed in the thermostat (Heating-block thermostat, model TK23, DITABIS, Germany) with set temperature to 37 °C. This procedure was repeated with four collagen samples in total from series of samples studying the effect of UV irradiation time and the effect of riboflavin concentration. The swelling properties were calculated using swelling ratio (SR) according to equation (1), where W_s is the weight of the swollen scaffold and W_d is the weight of the dry collagen scaffold. The water content was calculated according to equation (2). The results from the stability studies were processed in the software Excel.

$$\text{Swelling Ratio} = \frac{W_s}{W_d} \text{ [-]} \tag{1}$$

$$\text{Water Uptake} = \frac{(W_s - W_d)}{W_s} \cdot 100 \text{ [\%]} \tag{2}$$

4.5.4 Simulated physiological degradation

The simulated physiological degradation was carried out in the Solution A supplemented with collagenase in concentration of 2 $mg.l^{-1}$. The degradation studies were carried out according to following procedure. The vials were weighed with and without the dry collagen scaffolds to acquire dry collagen scaffold weight by subtraction. The scaffold was then immersed into the Solution A without collagenase, which was prepared prior to the measurement. After 1 hour the saline was sucked out of the vial using a syringe and the drenched collagen scaffold was weighted to acquire weight of swollen wet sample in the time interval of 1 hour. According to previous measurement of the stability, in 1 hour the scaffolds achieved equilibrium state and maintained stable swollen weight. Immediately after the samples were weighed, they were suffused in 1 ml of the Solution A with collagenase, which was prepared prior to the measurement. The samples were put in an incubator at 37 °C. The degraded samples were weighed after 1; 2; 4; 24; 48; 72; 96 hours. The degradation was calculated according to equation (3), where W_s is the weight of the swollen collagen scaffold at a certain time and W_{60} is the weight of the collagen scaffold after 60 minutes of swelling on the first day of measurement.

$$\text{Degradation} = \frac{W_s}{W_{60}} \cdot 100\ [\%] \tag{3}$$

We studied the degradation of all types of samples. The ones which were crosslinked in a solution of riboflavin with a constant concentration (1 000 μM) and irradiated for different time periods (5; 10; 15; 30 min.), and also the ones which were irradiated for the same amount of time (15 min.) but in riboflavin solutions with different concentrations (1; 10; 100; 1 000 μM).

4.5.5 Statistics

We have measured with a minimum of 4 samples of each type. The samples were statistically evaluated by calculating the standard deviations. For FTIR spectra, the spectra were obtained by averaging the spectra of 4 samples of one type. The statistical significance $p > 0,05$ was verified according to the t-test.

5 RESULT AND DISCUSSION

In this thesis, an alternative way to crosslink and produce stable porous collagen scaffolds avoiding the use of traditional EDC/NHS-based crosslinking method was investigated. The goal was to replace the toxic crosslinking agent with a natural alternative that would ensure the same strength of the crosslinking and solve the problem of cytotoxicity. A lot of possibilities were mentioned and discussed above. In this experiment, we chose riboflavin and UV radiation as a natural crosslinking agent because of its biocompatibility and ability to keep the mechanical structure of collagen intact. During the analysis of the prepared collagen scaffolds, we monitored the effect of riboflavin concentration, the time of irradiation, and the type of solvent. We used four concentrations of riboflavin solutions (1; 10; 100; 1 000 μM), four time periods of irradiation (5; 10; 15; 30 minutes), and two types of solvents (ultra-pure water, ethanol). During the crosslinking process two types of UV light were used. Longwave UV-A with the wavelengths of 315-400 nm and shortwave UV-C with the wavelengths of 200-280 nm.

5.1.1 Morphology and porosity evaluation

Collagen scaffolds used to be analyzed using scanning electron microscopy and optical microscopy. These methods, however, have multiple limitations. One of them is that it is only able to analyze the surface of the collagen scaffold. Furthermore, the preparation of the samples for the analysis could change the microstructure of the collagen sponge. For optical microscopy, the samples have to undergo various preparation processes too and have a big disadvantage called „out-of-focus-blur“ with thicker samples (greater than 10 μm).[78] This is why in this work the porosity and morphology of the collagen scaffolds were analyzed using CLSM.

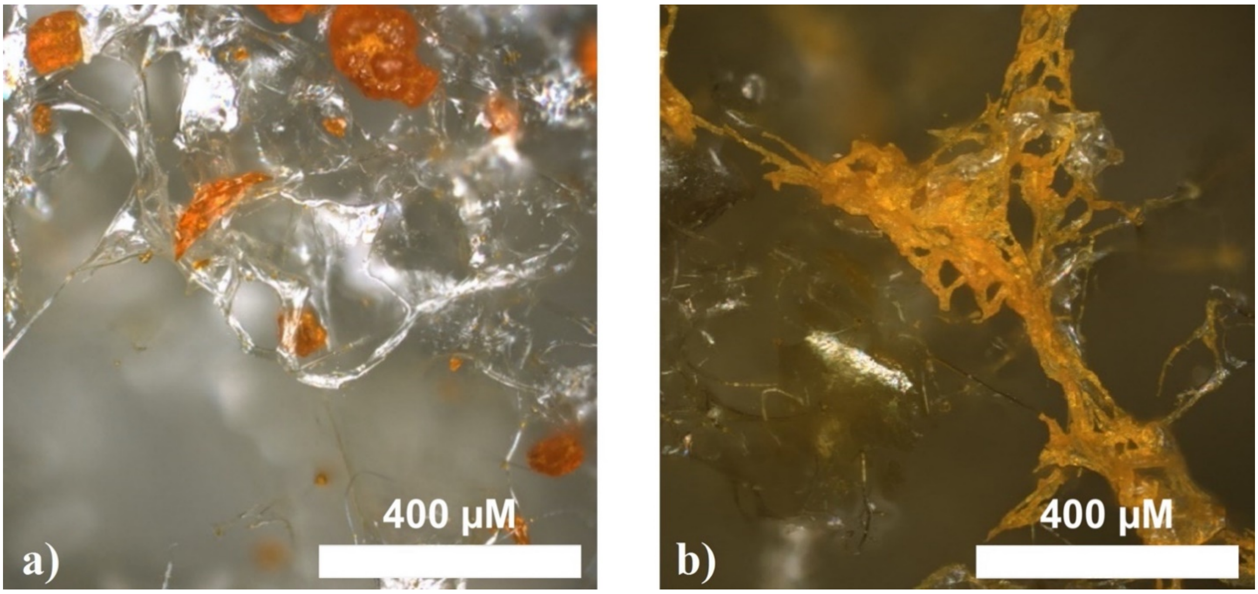

Figure 19 - CLSM analysis of collagen scaffolds a) crosslinked in 1 mM riboflavin and EtOH for 5 minutes b) crosslinked in 1 mM riboflavin and water for 5 minutes

The collagen scaffolds crosslinked with 1 mM riboflavin in water and ethanol were analyzed as first, to determine if there are any changes or differences in the morphology of the collagen scaffolds. The structure of the crosslinked collagen scaffolds can be seen in Figure 19. As one can see from the images there is one massive difference between the two methods of crosslinking. In the image Figure 19 (a) undissolved particles of riboflavin formed in the structure of the collagen fibers, due to its poor solubility in ethanol, can be observed. However, the image Figure 19 (b) depicts water-dissolved riboflavin covering the collagen fibers entirely creating a sort of coating. This may increase the efficiency of the crosslinking. The trapped riboflavin crystals can be considered as an undesirable characteristic, so no further attention was paid to it. Due to this fact, the ethanol-based crosslinking agent was abandoned, and the crosslinking was proceeded only with riboflavin dissolved in water.

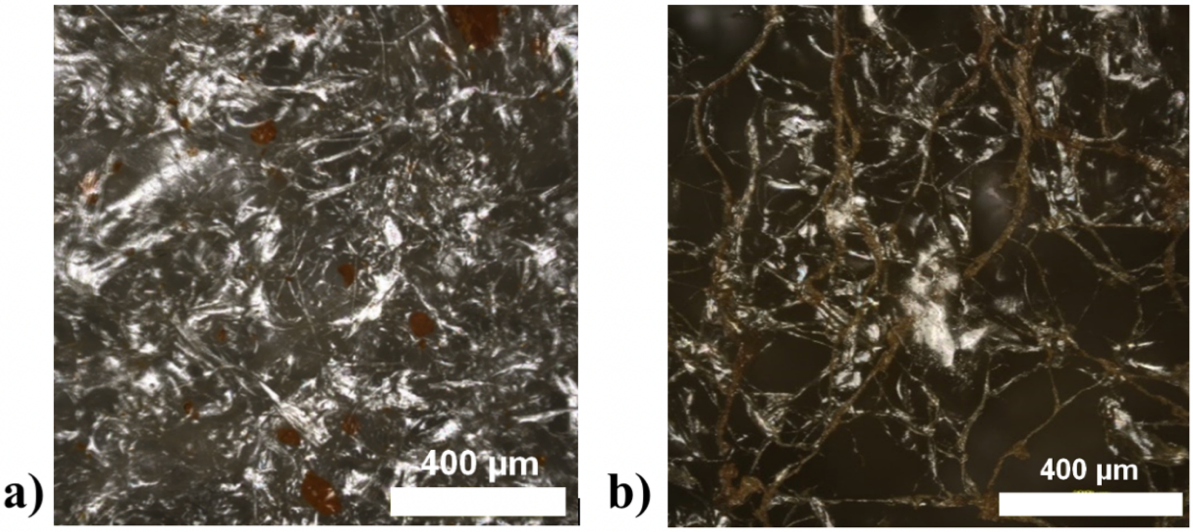

Figure 20 - Display of porous collagen structure a) crosslinked in 1 mM riboflavin and EtOH for 5 minutes b) crosslinked in 1 mM riboflavin and water for 5 minutes

The porous structure of the crosslinked collagen scaffolds can be seen in Figure 20. It can be seen that in the image Figure 20 (a) the porous system of the collagen structure is destroyed as opposed to the one crosslinked in riboflavin and water solution seen in the image Figure 20 (b). The pores of the collagen crosslinked in water were retained, and visibly bigger than the pores of the scaffolds crosslinked in ethanol. The pores of the scaffolds crosslinked in the alcoholic solution seem torn and crumpled while the ones crosslinked in water solution remain intact. The option of crosslinking in alcoholic solution was taken into consideration because collagen does not swell in ethanol and hydrolytic degradation is prevented. Nevertheless, these results further confirmed decision to abandon the crosslinking method in alcoholic solution of riboflavin. The referential scaffolds (non-crosslinked and crosslinked with EDC/NHS) are depicted in Figure 21.

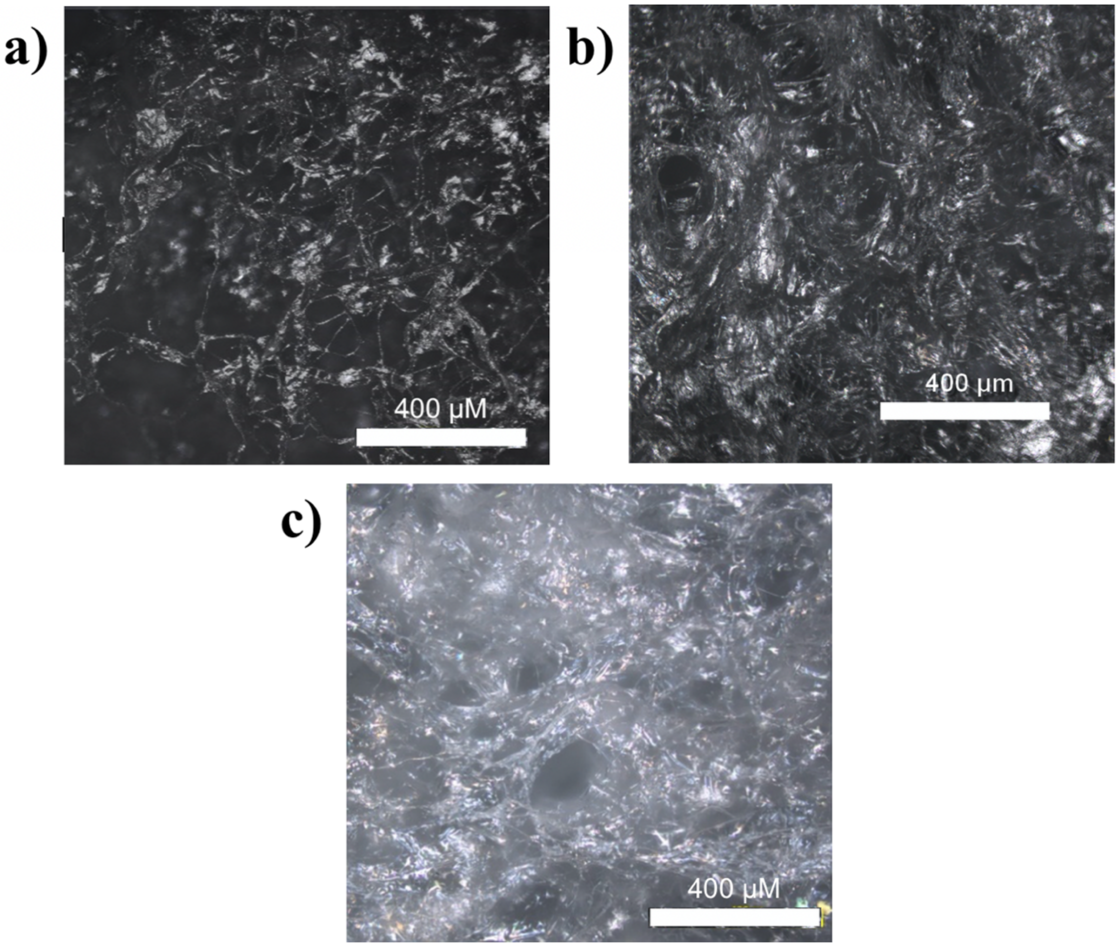

Figure 21 - Display of porous collagen structure a) crosslinked in EDC/NHS b) sufficed in EtOH (non-crosslinked) c) sufficed in ultra-pure water (non-crosslinked)

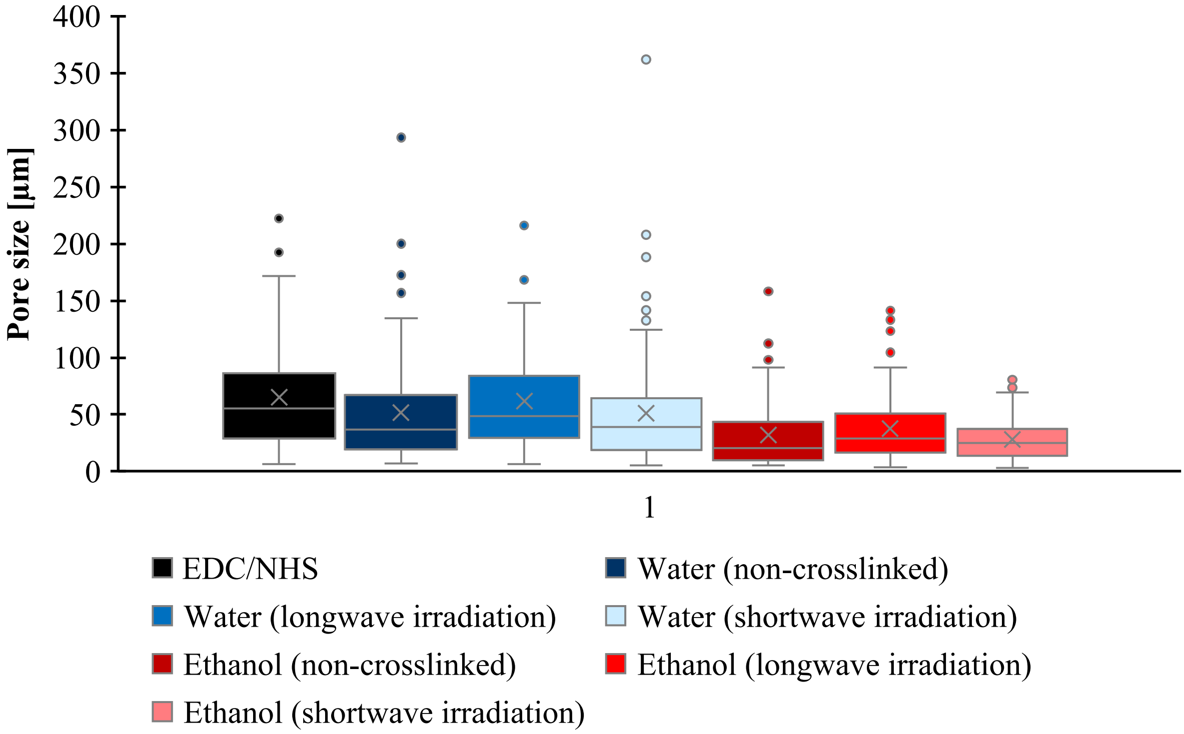

Figure 22 - Distribution of pore sizes

The obtained data were processed into a box plot that can be seen in Figure 22. The results show that the distribution of pores of scaffolds crosslinked in ethanol are too small and the pore sizes are around 50 µm. The scaffolds crosslinked in water, on the other hand, show similar pore size distribution as the scaffolds crosslinked in EDC/NHS which means that from the view of the porous structure the EDC/NHS crosslinking agent could be replaced by riboflavin dissolved in water followed by UV irradiation.

5.1.2 Evaluation of collagen structure and triple helical structure integrity

The secondary structure of collagen was analyzed and monitored using ATR-FTIR. For proper biological functionality of collagen, it is necessary to keep the secondary structure undamaged [79]. ATR-FTIR analysis was carried out for the qualitative determination of the changes in the secondary structure level. In Figure 23 we can see the expected types of band linkages present in pure collagen mass.

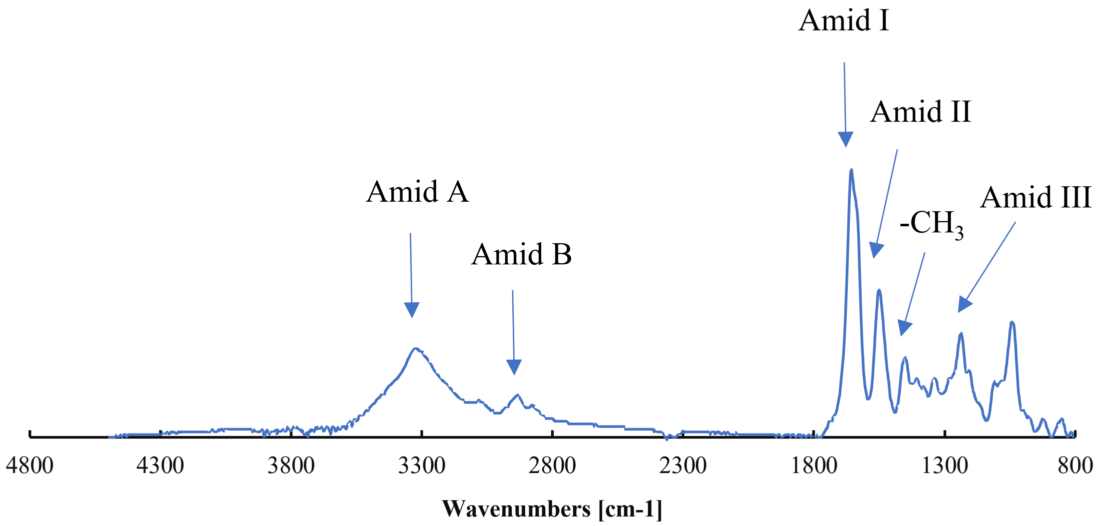

Figure 23 - Infrared spectrum of pure collagen

Pure collagen displays bands at 3 305; 2 959; 1 631; 1 544 and 1 237 cm^{-1}, which confirm the presence of the amide A, B, I, II, and III bands of collagen. These bands are characteristic for the peptide bond and also for collagen. The amide A is merged with the NH_2 stretching. The amide B peaks represent the CH_2-CH_3 stretching vibrations. The amide I absorption is due to the protein amide C=O stretching vibrations, the amide II absorption is the combination of amide N–H bending vibrations and C–N stretching vibrations. The amide III peaks are caused by 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.[80][81] Raw spectra from FTIR analysis were further processed for secondary derivative spectra analysis, which is known to uncover changes in secondary structure of proteins more sensitively [82].

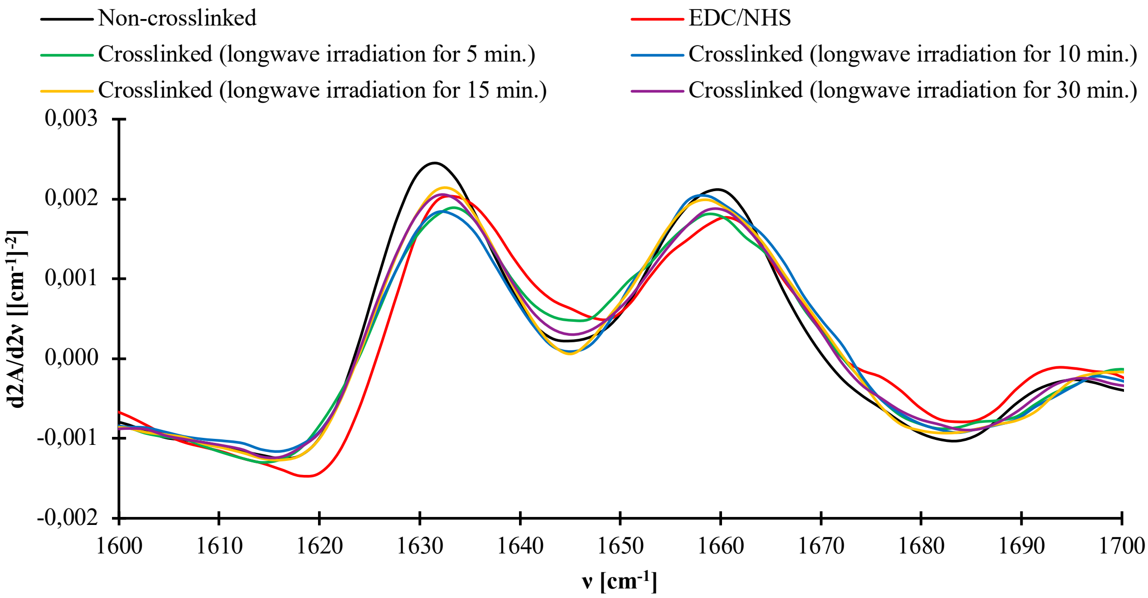

Figure 24 - Second derivation of FTIR spectra of Amide I band crosslinked at constant riboflavin concentration (1000 µM) and different time intervals (5; 10; 15; 30 min.) using longwave UV radiation

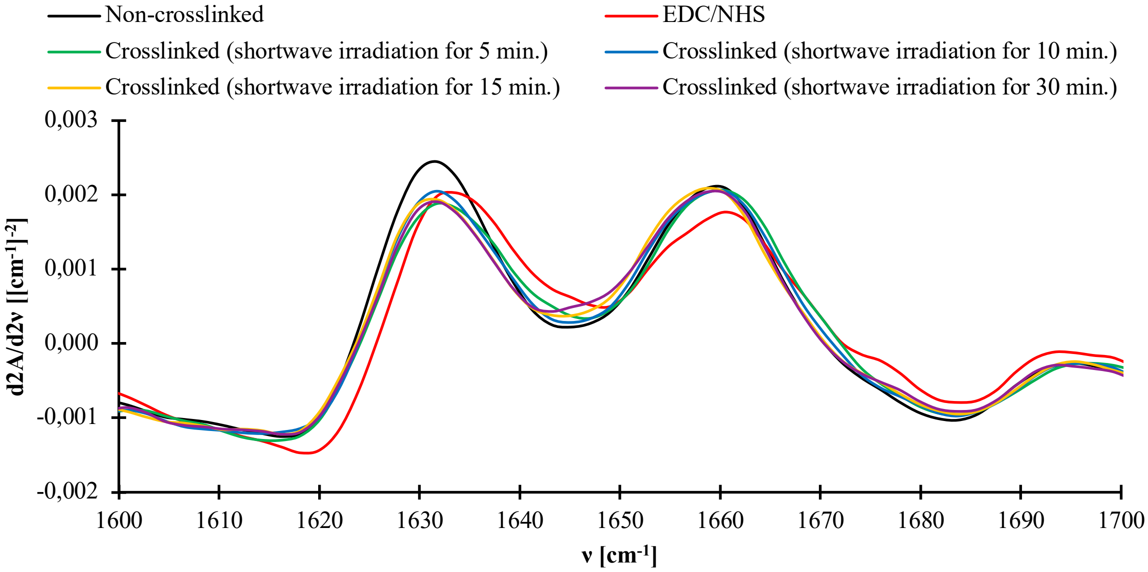

Figure 25 - Second derivation of FTIR spectra of Amide I band crosslinked at constant riboflavin concentration (1000 µM) and different time intervals (5; 10; 15; 30 min.) using shortwave UV radiation

The most sensitive peak areas for monitoring changes in the secondary structure of collagen are amide I, amide III, and the region of absorption of pyrrolidine groups (-CH_3) [82]. The second derivation of the amide I band was calculated in Origin Pro 8.5 for further confirmation of any secondary structure alterations. In Figure 24 and Figure 25 we can see the curves of the secondary derived amide I region of crosslinked collagen scaffolds irradiated in 4 different time intervals both longwave and shortwave, and non-crosslinked and crosslinked using EDC/NHS samples as well. In this case, it can be seen that the curves of both crosslinked in shortwave and longwave UV radiation did not affect the secondary structure of collagen. The curves of all analyzed samples adhere to the curve of the non-crosslinked specimen and also with the one crosslinked in EDC/NHS by minimal deviation. This finding shows promising results for a possible replacement of toxic crosslinking agents.

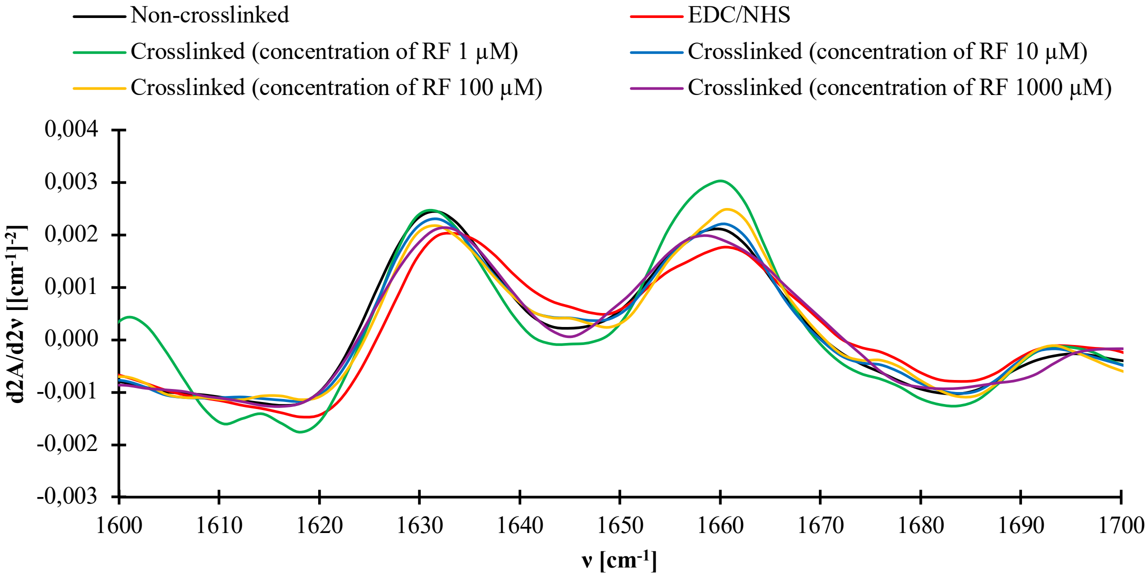

Figure 26 - Second derivation of FTIR spectra of Amide I band crosslinked at constant time period (15 min.) and different riboflavin concentrations (1; 10; 100; 1000 μM) using longwave UV radiation

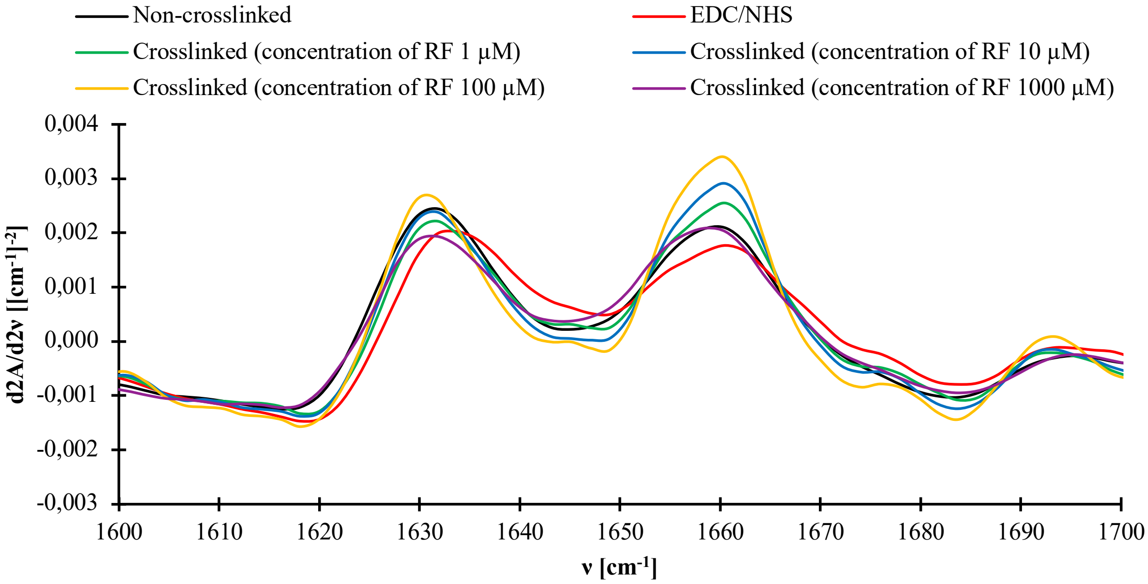

Figure 27 - Second derivation of FTIR spectra of Amide I band crosslinked at constant time period (15 min.) and different riboflavin concentrations (1; 10; 100; 1000 μM) using shortwave UV radiation

In Figure 26 and Figure 27 the curves of the amide I region of collagen scaffolds crosslinked using different concentrations of riboflavin agent both longwave and shortwave, and non-crosslinked and crosslinked with EDC/NHS as well can be observed. In this case of crosslinking, the changes and differences are more pronounced and recognizable. The curves that correspond to lower riboflavin concentrations deviate from the non-crosslinked collagen curve. This means that the secondary structure of collagen was somewhat altered. The samples crosslinked using longwave UV radiation show fewer changes, which could be explained by the fact that this radiation is low-frequency radiation in comparison to shortwave radiation. This means the shortwave radiation has more energy and therefore affected the secondary structure more. The interpretation of the results may suggest that with the decrease of the riboflavin concentration the alterations from the pure collagen curve increased. In both cases of crosslinking the highest riboflavin concentration, which is 1000 μM, seems to be acceptable and fairly similar to the non-crosslinked collagen curve, which could mean that this concentration was enough to protect the secondary structure. From the results we obtained we assume that the riboflavin acts as protectant from significant alteration of collagen secondary structure. The higher amount of the riboflavin was used during the crosslinking the more it shields and protects the collagen structure. It can be assumed that in the cases of 1; 10; and 100 μM riboflavin solution the concentration of riboflavin was too low for the UV radiation to just interact with riboflavin and form oxygen radicals. It not only interacted with riboflavin but also reacted with collagen causing its alterations in its secondary structure. Presumably, optimization of the riboflavin concentration high enough may be sufficient for maintaining the secondary structure of collagen and protecting any other additives accelerating wound healing (e.g. growth factors) that could be destroyed when exposed to UV radiation.

5.1.3 Stability in simulated physiological environment

The swelling properties of collagen scaffolds were studied in Solution A, a physiological solution with Ca^{2+} ions. In Figure 28 one can see the changes in the absorption capacity of collagen scaffolds after 60 minutes. These results were aquired from stability measurement of the samples that were crosslinked in a solution of riboflavin with a constant concentration (1 000 μM) but different irradiation time of both longwave UV-A and shortwave UV-C irradiation. As can be seen in the aforementioned figure, considering the standard deviations caused by inhomogenous porous structure, there was an absorption capacity reduction in all collagen scaffolds stabilised by UV radiation in comparison to the non-crosslinked scaffolds. The reduction of absorption capacity is also visible in collagen scaffolds crosslinked by EDC/NHS, which may suggest that the stabilisation of the structure occured. It can be expected that the decrease of absorptive properties of the material is related to the formation of new covalent bonds during crosslinking that creates a more compact and more complex structure, which is less prone to absorbe and bond water molecules from aqueous solutions. The closest absorption capacity to the EDC/NHS-crosslinked scaffolds was achieved in the scaffolds irradiated by the shortest irradiation time for both longwave UV-A and shortwave UV-C light. The other scaffolds irradiated for 10; 15; and 30 minutes all showed a decrease in absorption capacity but not as low as the referential chemical crosslinking method using EDC/NHS. From these results, it may be assumed that the possible option to replace the conventional crosslinking method would be the five-minute irradiation method, although other samples showed a decrease in absorption capacity it was not sufficient enough to achieve the EDC/NHS crosslinking level.

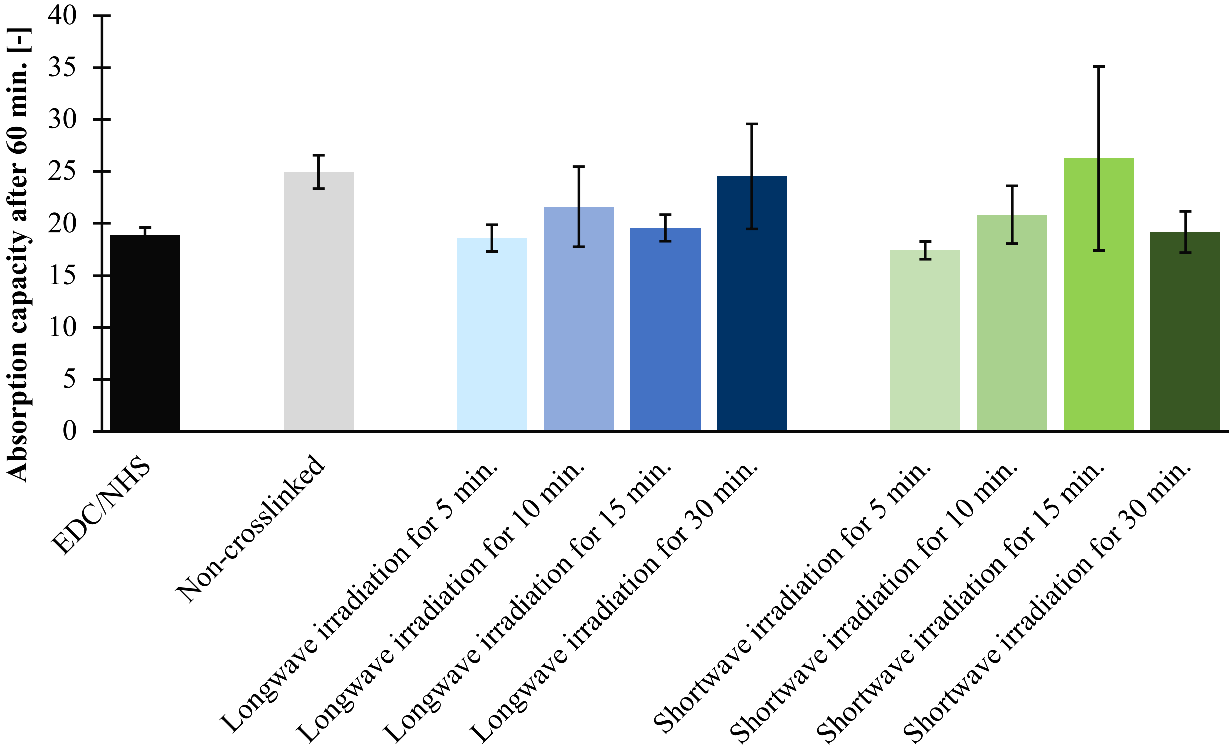

Figure 28 - Swelling properties of collagen scaffolds in PBS (crosslinked at different time intervals)

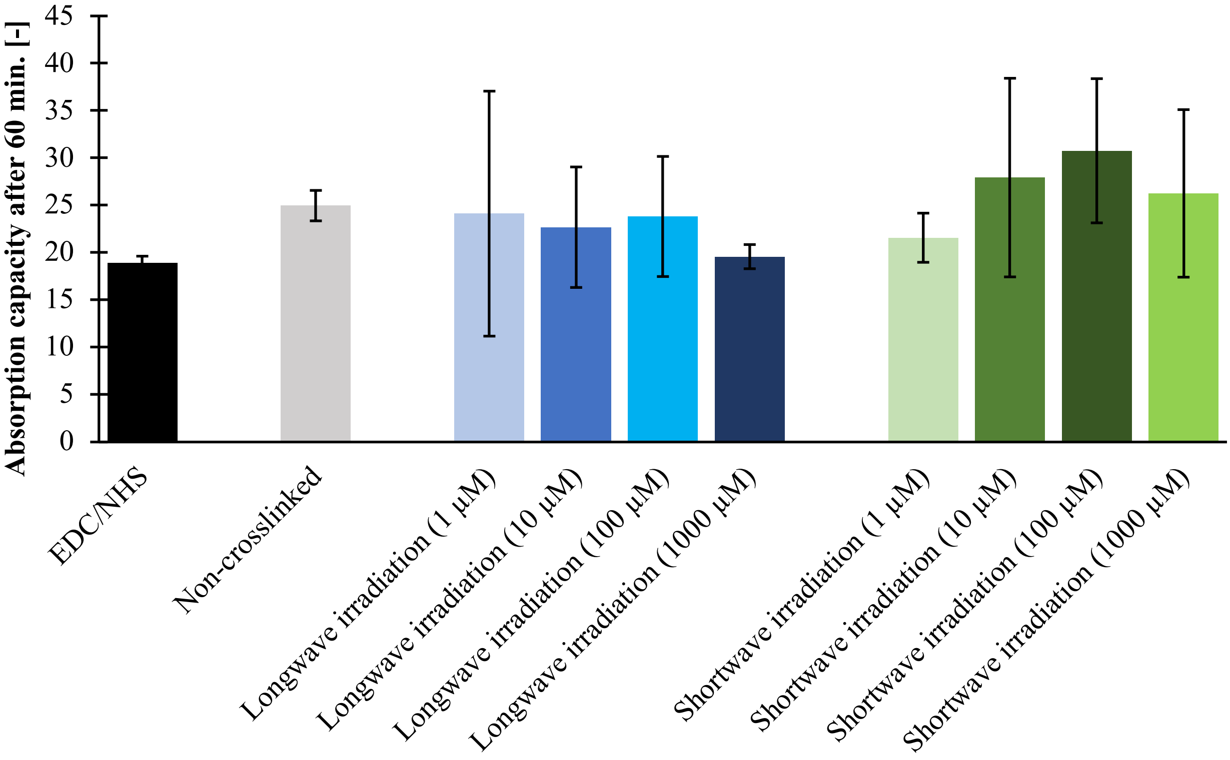

Figure 29 - Swelling properties of collagen scaffolds in PBS (crosslinked at different riboflavin concentration)

In Figure 29 you can see the changes in absorption capacity of the collagen samples after 60 minutes. These results were aquired from stability measurement of the scaffolds that were crosslinked in a solution of riboflavin with different concentrations but constant irradiation time (15 min.) of both longwave UV-A and shortwave UV-C irradiation. As can be seen in Figure 29 all the samples crosslinked with longwave UV-A showed reduction in absorption capacity in comparison to the non-crosslinked collagen scaffolds. It can be assumed that a stablilization took place in those cases. In the case of the scaffolds crosslinked using shortwave UV-C the reduction was not satisfactory enough. The absorption capacity level of the samples crosslinked in 10 and 100 μM riboflavin solution did not exceed the levels of the non-crosslinked scaffolds. The other two crosslinked in 1 and 1 000 μM suggest that stabilization may have took place but not sufficient enough to replace the EDC/NHS method. The scaffolds crosslinked in 1 000 μM riboflavin solution using longwave UV-A met the level of the scaffolds satbilized in EDC/NHS. The other samples irradiated with longwave UV-A all showed a decrease in the absorption capacity but the samples crosslinked in the highest concentration of riboflavin were the most significant. From these results, it may be assumed that the possible option to replace the conventional crosslinking method would be the use of 1 000 μM riboflavin solution, although other samples showed a decrease in absorption capacity it was not sufficient enough to achieve the EDC/NHS crosslinking level.

5.1.4 Simulated physiological degradation

The degradation of the prepared collagen scaffolds was simulated in an incubator in PBS at 37°C. The degradation itself was expressed as the mass loss of the samples. Figure 30 and Figure 31 shows the mass loss of the collagen scaffolds irradiated for different time intervals. As can be seen in Figure 30 and Figure 31 the slowest degradation rate belongs to the samples crosslinked using EDC/NHS. In the beginning the other samples showed similar rates as the collagen samples crosslinked with EDC/NHS. All the prepared collagen scaffolds irradiated for different time had slower degradation rates as the non-crosslinked collagen scaffolds, but not as low as the scaffolds crosslinked in EDC/NHS. In Figure 32 and Figure 33 the mass loss of collagen scaffolds crosslinked in different concentrations of RF solution is depicted. The results were similar to the ones depicted in Figure 30 and Figure 31. All the prepared collagen scaffolds showed lower degradation rate as the non-crosslinked sample but not as low as the samples crosslinked in EDC/NHS. Some of the samples showed higher mass than 100 %. It can be assumed that this occurred due to the fact that during the degradation the bonds between the collagen fibers started to break and it caused its further swelling. We appointed these results unsatisfactory, even though every type of sample demonstrated lower degradation rate compared to the non-crosslinked scaffolds, none of the samples overtake the rate of the samples crosslinked using EDC/NHS. This means that there was less stabilization during the crosslinking process than in the case of EDC/NHS. Therefore, the goal to replace the EDC/NHS crosslinking method could not be achieved with the same efficiency. It can be assumed that the stabilization did not occur because of the remaining riboflavin in the system. Riboflavin is present in the form of phosphate salt that has the ability to destroy covalent bonds and therefore support the degradation. This could cause the fast reduction and high degradation rate of the crosslinked scaffolds. This problem could be solved by washing out the remaining riboflavin from the samples. This way higher stabilization could be achieved.

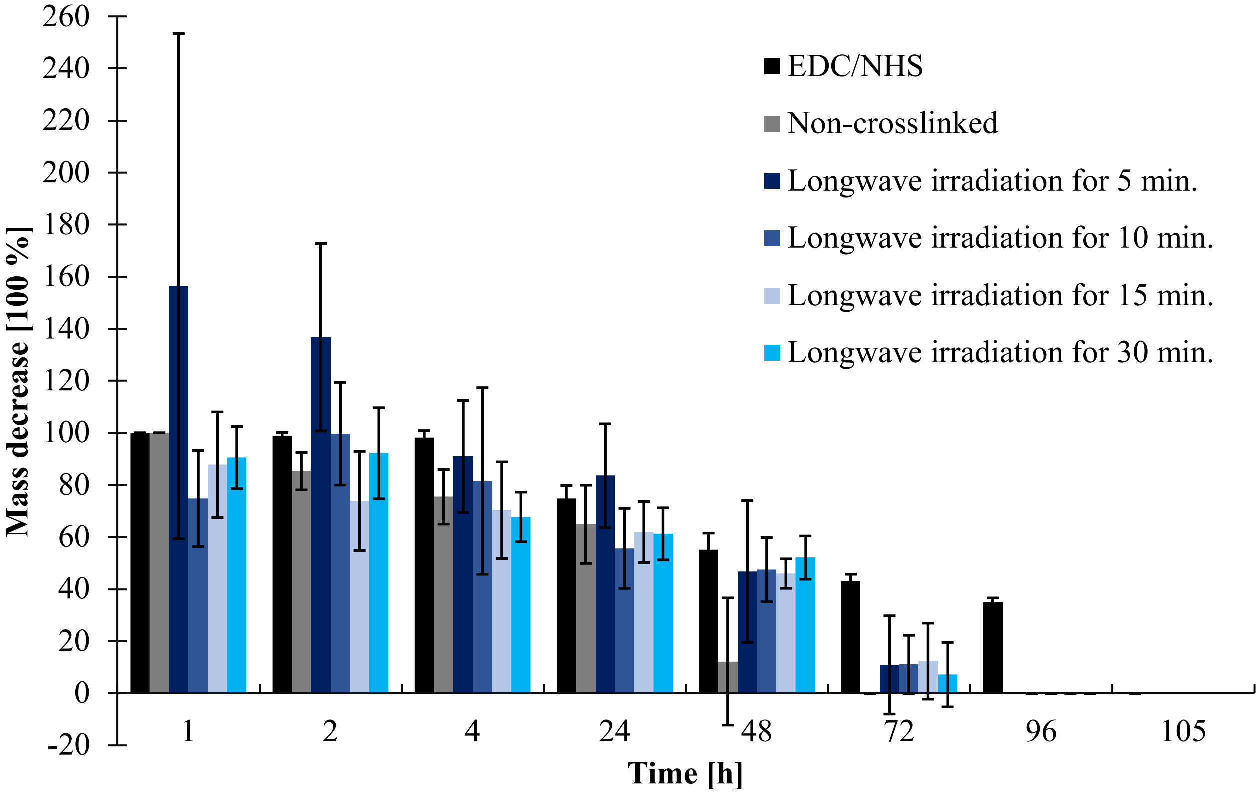

Figure 30 - Degradation of collagen scaffolds (crosslinked at different time intervals using longwave UV-A)

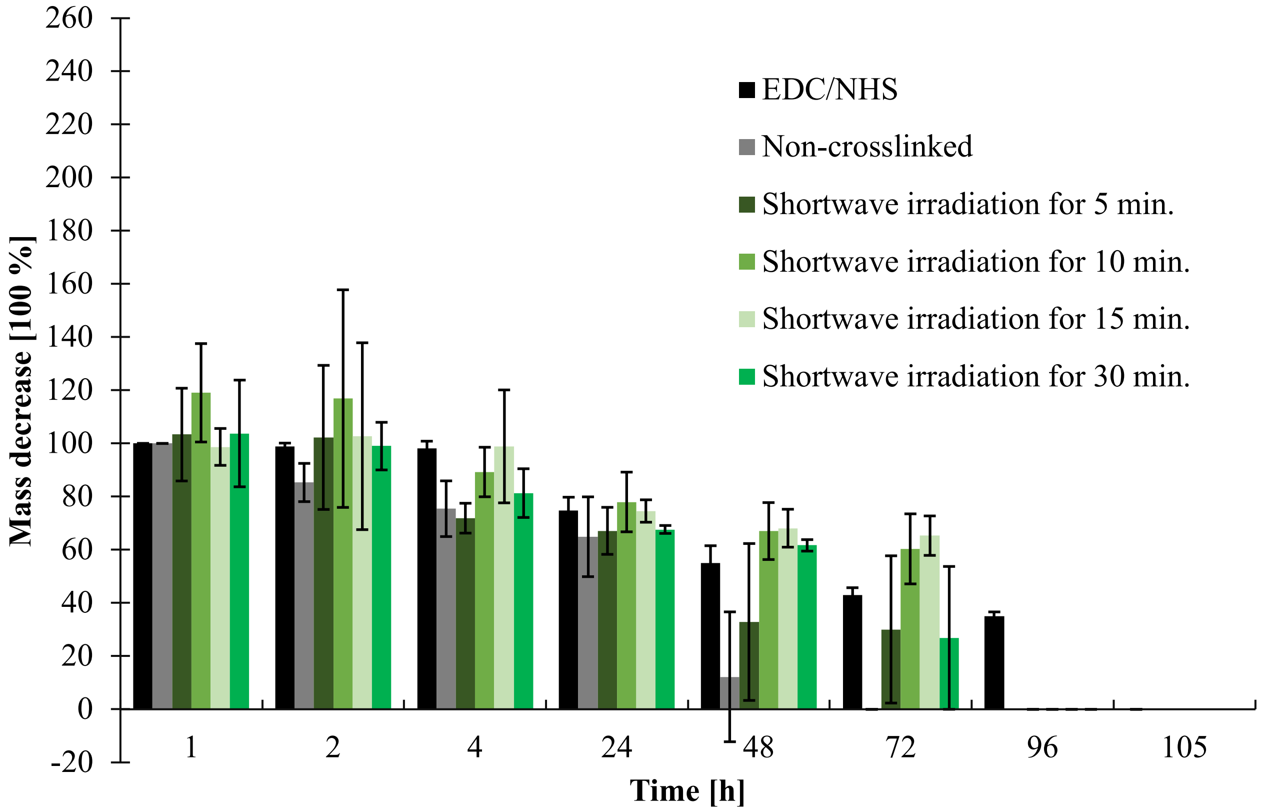

Figure 31 - Degradation of collagen scaffolds (crosslinked at different time intervals using shortwave UV-C)

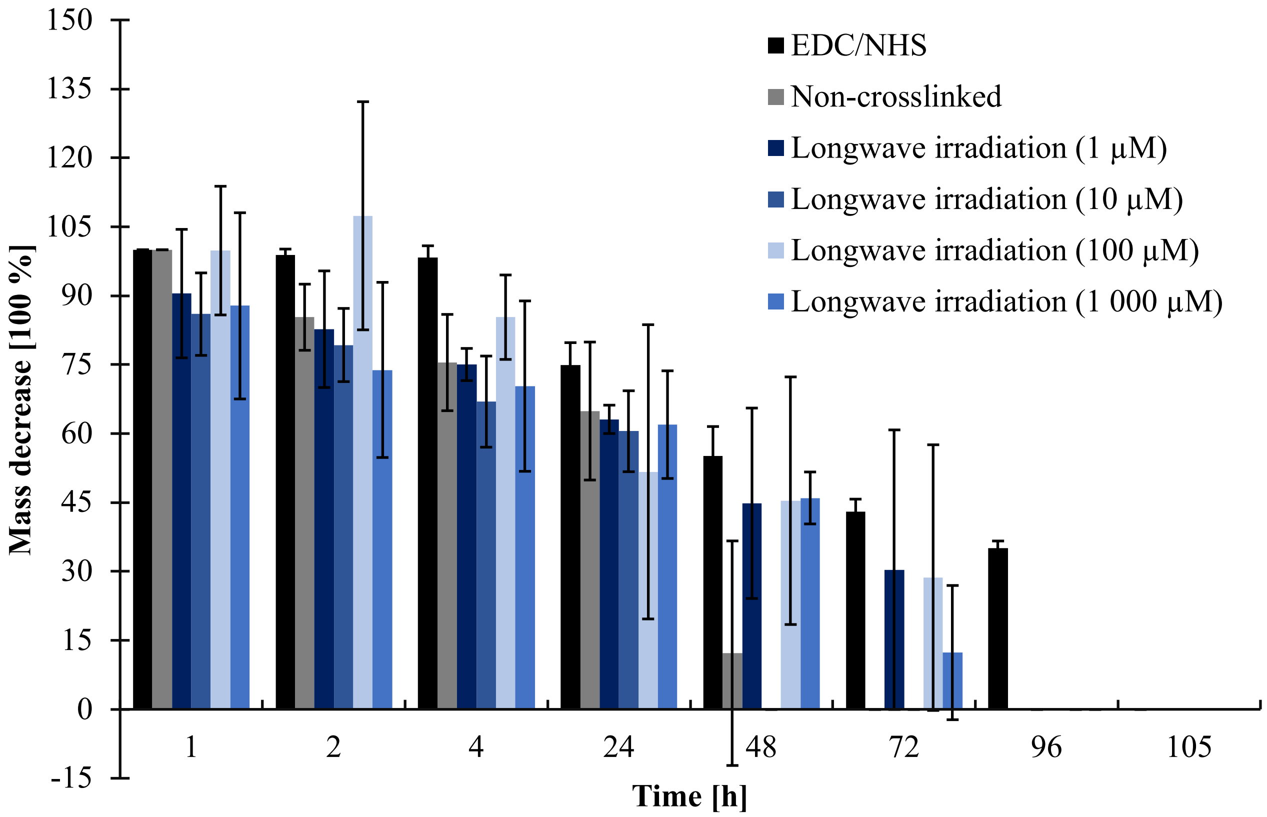

Figure 32 - Degradation of collagen scaffolds (crosslinked in different concentrations of RF solution using longwave UV-A)

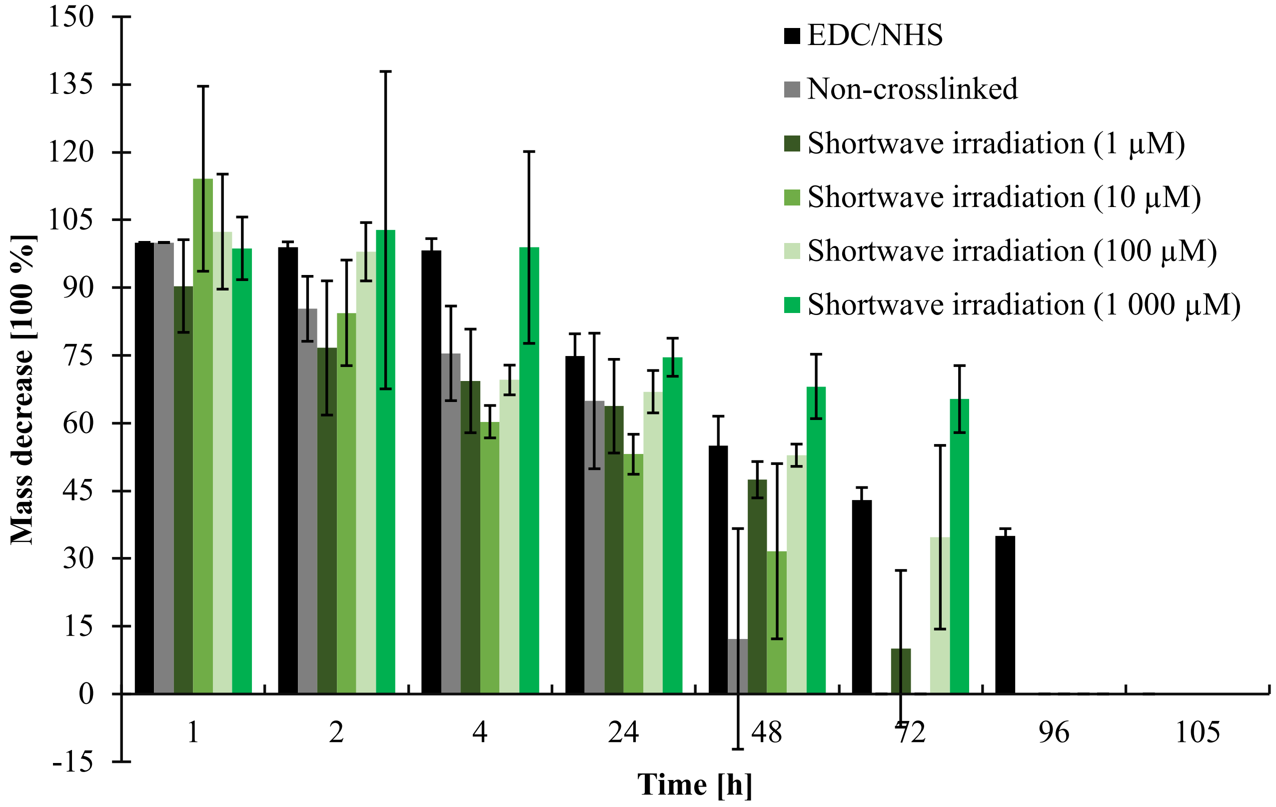

Figure 33 - Degradation of collagen scaffolds (crosslinked in different concentrations of RF solution using shortwave UV-C)

6 CONCLUSION

The goal of the work lies in the preparation of collagen scaffolds followed by their crosslinking using a non-toxic, biocompatible, natural crosslinking agent - riboflavin. As a mediator for the crosslinking procedure, longwave UV-A and shortwave UV-C were used securing the non-toxicity of the whole process. For the study and characterization of the crosslinked collagen scaffolds various methods were used, such as gravimetrical measurement of swelling and degradation, evaluation of chemical structure by FTIR and morphology observation by CLSM. Each method evaluates different aspects and properties of the collagen samples.

As a conclusion based on the results from morphology and porosity measurements of the samples, it seems that the right solvent for the crosslinking would be ultra-pure water, because alcoholic RF solution created scaffolds with damaged porous system. The pore sizes of the mentioned scaffolds were also unsatisfactory. Therefore, further in our experiments the collagen scaffolds were strictly crosslinked in aqueous RF solutions. In terms of the secondary structure, which was evaluated in terms of ATR-FTIR, the result showed that the concentration of the RF solution used during the crosslinking highly influences the finished product. The use of the highest RF concentration (1 000 μM) seemed to be optimal for the crosslinking via both for longwave UV-A and shortwave UV-C. The samples crosslinked by this way exhibited less alterations in their secondary structure and were the most similar to the non-crosslinked collagen samples. The time of irradiation affects the secondary structure of collagen and also the quality of the prepared scaffolds way less than the concentration of RF present in the crosslinking agent. In the case of the swelling properties, the most promising and similar results compared to the EDC/NHS method were obtained from the samples crosslinked using longwave UV-A. The scaffolds crosslinked in different RF concentrations and for different time intervals with longwave UV-A all showed similar reduction in absorption capacity like the scaffolds crosslinked with EDC/NHS. In conclusion from the point of swelling properties the longwave UV-A irradiation method seems to be the most favorable. Even though in the case of degradation all the samples had better degradation rates than the non-crosslinked scaffolds, however, the results were not sufficient enough to reach the level of stability provided by EDC/NHS. Better results were obtained for the scaffolds crosslinked with shortwave UV-C, which were not as good as the results obtained for the EDC/NHS crosslinking method.

Although EDC/NHS crosslinking treatment is able to stabilize the sample better, even 3-4 days degradation of the scaffolds are enough for the sufficient use of these scaffolds. Collagenous scaffolds with shorter disintegration period may be useful in treating infected wounds, where a relatively quickly disintegrating scaffold loaded with antibacterial agents may release these substances more effectively than the stable one. Moreover, biocompatibility of the crosslinking treatment outweighs the missing stability for longer time. Thus EDC/NHS values don't always have to be achieved and sometimes it is not even necessary

In conclusion, the RF crosslinking methods offers a possible way to crosslink collagen scaffolds but not as effective as the EDC/NHS option. Possible difficulty that could cause some unfulfilling results could be the fact that the biomaterials had to be preserved at 37 °C most of the time. During some analysis minor temperature changes could occur that would alter the results slightly. The laboratory environment is different from the natural environment of the biomaterials used during the experiment therefore the conditions must be reproduced as best as possible. For the complete replacement of the conventional method of collagen crosslinking further studies and optimization of conditions is needed.

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8 LIST OF ABBREVIATIONS

CA	Citric acid
CAD	Citric acid derivative
CLSM	Confocal laser scanning microscopy
DHT	Dehydrothermal treatment
EDC	N-(-3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FTIR	Fourier-transformed infrared spectroscopy
GA	Glutaraldehyde
GE	Genipin
LA	L-Arginine
LO	Lysyl oxidase
MMPs	Matrix metalloproteinases
NHS	N-hydroxysuccinimide
PBS	Phosphate buffer saline
PDA	Polydopamine
RM	Regenerative medicine
SR	Swelling ratio
TA	Tannic acid
TE	Tissue engineering
UV	Ultraviolet
UV-A	Long wave ultraviolet light
UV-C	Short wave ultraviolet light
UVT	Ultra-violet treatment

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