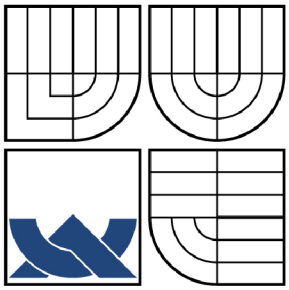

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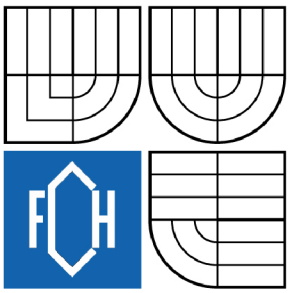

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

COLLAGEN CROSS-LINKING USING OXIDIZED CELLULOSE

SÍŤOVÁNÍ KOLAGENU POMOCÍ OXIDOVANÉ CELULÓZY

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

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BRNO 2009

Vysoké učení technické v Brně
Fakulta chemická
Purkyňova 464/118, 61200 Brno 12

Zadání diplomové práce

Číslo diplomové práce:	**FCH-DIP0320/2008**	Akademický rok:	**2008/2009**
Ústav:	Ústav chemie materiálů		
Student(ka):	**Bc. Pavel Filka**		
Studijní program:	Chemie, technologie a vlastnosti materiálů (N2820)		
Studijní obor:	Chemie, technologie a vlastnosti materiálů (2808T016)		
Vedoucí diplomové práce:	**Ing. Lucy Vojtová, Ph.D.**		
Konzultanti diplomové práce:			

Název diplomové práce:

Síťování kolagenu pomocí oxidované celulózy

Zadání diplomové práce:

Využití karboxylů oxidované celulózy k síťování kolagenu obsahující volné aminoskupiny. Navržením správného poměru mezi kolagenem a celulózou by se mělo dosáhnout kompletního zesíťování (nezbydou žádné volné aminoskupiny na kolagenu) a zaručit tak řízenou degradaci daného materiálu.
1. Literární rešerše na téma síťování kolagenu, oxidovaná celulóza, aktivace karboxylových skupin a jejich reakce s aminokyselinami.
2. Experimntální práce:
a) aktivace karboxylových skupin systémem EDC/NHS.
b) Reakce komplexu celulózy s aminokyselinami.
c) pomocí získaných výsledků stanovit minimální potřebnou koncentraci želatiny nebo kolagenu pro dokonalé sesíťování.
d) reakce kolagenu s aktivovanou oxidovanou celulózou - v různých poměrech obou složek.
3. Interpretace vztahu mezi strukturou směsi kolagen - celulóza (želatina - celulóza) a jejími vlastnostmi.
4. Závěr

Termín odevzdání diplomové práce: 22.5.2009

Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Bc. Pavel Filka	Ing. Lucy Vojtová, Ph.D.	prof. RNDr. Josef Jančář, CSc.
Student(ka)	Vedoucí práce	Ředitel ústavu

V Brně, dne 1.10.2008

doc. Ing. Jaromír Havlica, DrSc.
Děkan fakulty

SUMMARY

Theoretical part of submitted diploma thesis deals with the basic finding of collagen, one of the most representing proteins in a human body, and oxidized cellulose used in medicine for several decades. Main part of the literature search is concerned on the collagen cross-linking, which is necessary for the collagen stability improving, thereby increasing the resistance against its degradation. As a cross-linking agent, the oxidized cellulose having the haemostatic properties as well as the carboxyl functional groups suitable for cross-linking proteins can be used. Experimental work was focused on the investigation of mutual behaviors of mixed water solutions based on oxidized cellulose and collagen. Films or lyophilized sponges prepared from these polymer mixtures could be used in medicine as a haemostatic or antibacterial wound healing coverings.

The series of weight ratios between collagen and oxidized cellulose (9:1, 3:1, 5:3, 1:1, 1:2, 1:3, 1:9) involving constant content of collagen and increasing amounts of cellulose were prepared. Their ability to chemical linking, generating the formation of amide bond between carboxyl group of oxidized cellulose and free amino group of collagen, was investigated via two UV-VIS spectroscopic methods using a colored reaction among the chemical reagent (ninhydrin or 2,4,6-trinitrobenzenesulfonic acid) and free amino groups of collagen. Moreover, the carboxyl groups of oxidized cellulose were activated either in the polymer solution (in situ) or in the form of a film using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide (EDC/NHS). The changes at the secondary structure level were investigated by Fourier-transformed infrared spectroscopy (FT-IR). The stability of thin prepared films from each polymer mixture was determined using hydrolytic degradation at 37 °C. Morphological changes in the two types of samples, one frozen quickly at – 196 °C and second slowly at -30 °C, were observed using scanning electron microscopy (SEM).

During the polymer mixture preparation, the precipitation up to the ratio of 1:1 between collagen and cellulose has occurred. UV-VIS analyses confirmed free amino groups reduction resulting in cross-linking between collagen and cellulose as well as increasing in hydrolytical stability obtained from degradation measurement of prepared films. At the ratio higher than 1:2 up to 1:9 the polymer mixture was homogeneous without precipitation, however, from the increasing free amino groups it is presupposed that cellulose acted as physical cross-linker at the small amount and beyond the equilibrium state works more like solvent. It probably caused the changes in collagen at the secondary structure level registered from FT-IR analysis. Activation of cellulose carboxyl groups by EDC/NHS was not confirmed.

Collagen to cellulose ratio affected as well as the porosity and the pore size of prepared scaffolds. Based on SEM, the porosity of scaffolds froze by liquid nitrogen was between 46 – 60 % up to the ratio of 1:1 and significantly increased with cellulose addition up to 81 % (at ratio of 1:9). Average pore size of neat collagen was very small (14 ± 5 μm) in comparison with pure oxidized cellulose (79 ± 24 μm). That is why the cellulose addition increased the pore size approximately up to 55 μm except for the 1:9 ratio having very large pore size

(186 ± 76 μm) and very regular structure resembling honeycomb seen at pure cellulose as well.

Keywords: Collagen, oxidized cellulose, cross-linking

SOUHRN

Předložená diplomová práce v teoretické části shrnuje základní fakta o kolagenu, bílkovině, která je nejrozšířenější v lidském organismu a oxidované celulóze používané v medicíně po několik desetiletí. Hlavním tématem této časti je síťování kolagenu, které je důležitým faktorem pro stabilizaci kolagenu podporující odolnost proti jeho degradaci. Jako síťující činidlo lze použít právě oxidovanou celulózu, která má kromě hemostatického účinku i funkční karboxylové skupiny vhodné k síťování proteinů. Praktická část práce byla zaměřena na sledování vzájemného chování směsí roztoků oxidované celulózy a kolagenu. Filmy či pěnové lyofilizáty připravené z těchto polymerních směsí by mohly sloužit jako účinná hemostatika nebo jako antibakteriální krytí ran podporující hojení.

Byla zvolena řada hmotnostních poměrů mezi kolagenem a oxidovanou celulózou (9:1, 3:1, 5:3, 1:1, 1:2, 1:3, 1:9) se zachováním konstantního množství kolagenu, ale se vzrůstajícím množstvím celulózy. Jejich schopnost chemicky se vázat a umožnit tak vznik amidových vazeb mezi volnými aminovými skupinami kolagenu a karboxylovými skupinami oxidované celulózy byla sledována pomocí dvou UV-VIS spektroskopických metod, které využívají barevné reakce chemického činidla (ninhydrinu či 2,4,6-trinitrobenzensulfonové kyseliny) se zbylými volnými aminovými skupinami kolagenu. Karboxylové skupiny oxidované celulózy byly navíc aktivovány jak v polymerním roztoku tak i ve formě filmu směsí činidel 1-ethyl-3-(3-dimethylaminopropyl)karbodiimidu a N-hydroxysukcinimidu (EDC/NHS). Pomocí infračervené spektroskopie s Fourierovou transformací (FT-IR) byly vyšetřeny změny vzorků na úrovni sekundární struktury kolagenu. Stabilita připravených směsí byla sledována ve formě filmu pomoci hydrolytické degradace při 37 °C. Morfologické změny na dvou typech lyofilizovaných vzorků, vymražených rychle při -196 °C nebo pomaleji při -30 °C, byly sledovány pomocí rastrovací elektronové mikroskopie (SEM).

Při přípravě polymerních směsí se obě složky (kolagen i celulóza) se vzrůstajícím obsahem celulózy srážely až do poměru 1:1. UV-VIS analýzy potvrdily pokles volných $–NH_2$ skupin poukazující na síťování kolagenu s celulózou shodně s nárůstem odolnosti vůči hydrolytické degradaci získané z měření úbytků hmotností připravených filmů. Od poměru 1:2 se složky již nesrážely, polymerní roztok byl homogenní, ale z důvodu nárůstu počtu volných aminových skupin od tohoto poměru výše lze usoudit, že celulóza fungovala v malém obsahu pouze jako fyzikální síťovalo a po dosažení rovnovážného stavu s kolagenem funguje spíše jako rozpouštědlo. Tímto způsobem zřejmě mohlo dojít ke změnám kolagenu až na úrovni sekundární struktury zaznamenané pomocí FT-IR. Aktivace karboxylových skupin celulózy činidly EDC/NHS nebyla prokázána.

Poměry složek ovlivnily i porózitu a velikosti pórů připravených lyofilizátů určených pomocí SEM. Do poměru 1:1 byla porózita skafoldů vymražených kapalným dusíkem mezi 46 – 60 %, po dalším přidání celulózy stoupla až na 81 % (u poměru 1:9). Průměrná velikost pórů samotného kolagenu byla velice malá (14 ± 5 μm), oproti oxidované celulóze (79 ± 24 μm), proto přídavek celulózy vždy zvýšil velikost pórů na cca 55 μm s výjimkou poměru 1:9,

mající vysokou průměrnou velikost pórů (186 ± 76 μm) a velmi pravidelnou strukturu připomínající včelí plást, kterou má i samotná celulóza.

Klíčová slova: kolagen, oxidovaná celulóza, síťování.

FILKA, P. Collagen cross-linking using oxidized cellulose. Brno, 2009. 94 p. Diploma thesis, Brno university of Technology, Faculty of chemistry, Institute of Materiále Science. Supervisor Ing. Lucy Vojtová, Ph.D.

PROHLÁŠENÍ

Prohlašuji, že jsem diplomovou práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Diplomová 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.

.....
podpis diplomanta

DECLARATION

I declare that this thesis has been compiled by my self and on my own and I cited all my information sources completely and correctly. The diploma thesis is in terms of its contents a property of the BUT Faculty of chemistry and its usage for commercial purposes is subject to a prior consent of the supervisor and the dean.

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author's signature

Acknowledgements:

I would like to thank my supervisor Ing. Lucy Vojtová, Ph.D. for providing me with all the important prerequisites to my work and for her gentle patience and for invaluable guidance on my writing, prof. RNDr. Josef Jančář, CSc. for valuable advises and all colleagues involved in this work.

1 INTRODUCTION

Collagen, a well-known protein, has been widely used in medical applications. Many natural polymers and their synthetic analogues are used as biomaterials, but the characteristics of collagen as a biomaterial are distinct from those of synthetic polymers mainly in its mode of interaction in the body. Collagen exhibits biodegradability, weak antigenecity and superior biocompatibility compared to other natural polymers, such as albumin and gelatin [1].

The primary reason for the use of collagen in biomedical applications is that collagen can form fibers with extra strength and stability through its self-aggregation and cross-linking. In most of drug delivery systems made of collagen, in vivo absorption of collagen is controlled by the use of cross-linking agents, such as glutaraldehyde, chromium tanning, formaldehyde, polyepoxy compound, acylazide, carbodiimides, and hexamethylenediisocyanate. Physical treatment, such as ultra-violet, gamma-ray irradiation and dehydrothermal treatment have been efficiently used for the introduction of cross-links to collagen matrix as well [1, 2].

The main goal of this work is cross-linking collagen using oxidized cellulose. Oxidized cellulose is used in medical devices such as absorbable haemostatic agents and absorbable adhesion barriers and furthermore in cosmetics, food production etc.. Oxidized cellulose has substituted carboxyl groups that can be used for cross-linking and stabilization of collagenous matrix and thus replace toxic chemical cross-linking agents. In order to increase the efficiency of the reaction between carboxyl groups of oxidized cellulose and amino groups of collagen, the carboxyl groups were activated both in situ and in the form of air-dried films using carbodiimide compound. Prepared polymer mixtures were analyzed in term of the change in chemical structure via UV-VIS and FT-IR analyses, the polymer films were evaluated with a view of hydrolytical stability (biodegradability) at 37 °C and freeze-dried sponges were analyzed by SEM of purpose to see the changes in the samples' morphology assessed as the sponges' porosity and pore size.

2 CURRENT STATE OF THE ART

2.1 Collagen

Collagen represents a large family of structurally related proteins and it is the most abundant molecule in vertebrates [2]. It can be found in large quantities in tendon, bone, skin, cornea and cartilage [3].

Collagen is a renewable resource which is synthesized in the body by fibroblasts. That is the reason why there is a tendency leading to innovation of its production and searching for new applications of its use [4]. The unique characteristic of collagen is its triple helical structure. It is stabilized by vast number of intra- and intermolecular hydrogen's bonds [5].

The main applications of collagen as drug delivery systems are collagen shields in ophthalmology, sponges for burns/wounds, mini-pellets and tablets for protein delivery, gel formulation in combination with liposomes for sustained drug delivery, as controlling material for transdermal delivery, and nanoparticles for gene delivery and basic matrices for cell culture systems. It is frequently used for tissue engineering including skin replacement, bone substitutes, and artificial blood vessels and valves as well [1].

About 22 different types of collagen have been described so far. Currently, at least 13 types of collagen have been isolated which vary in the length of the helix, size of the non-helical portions and the nature (Table 1) [6]. Type I collagen is predominant in higher order animals especially in their skin, tendons and bones where extreme forces are transmitted [7].

2.1.1 Structure and constitution

The basic collagen molecule contains three polypeptide α-chains (Figure 1), each consisting of more than 1000 amino acids which are linked by the amide bonds. These three polypeptide strands are wrapped around each other forming right-handed superhelix with a length of 300 nm and diameter of 1.5 nm. Molecular weight is approximately 300 000 g.mol^{-1}.

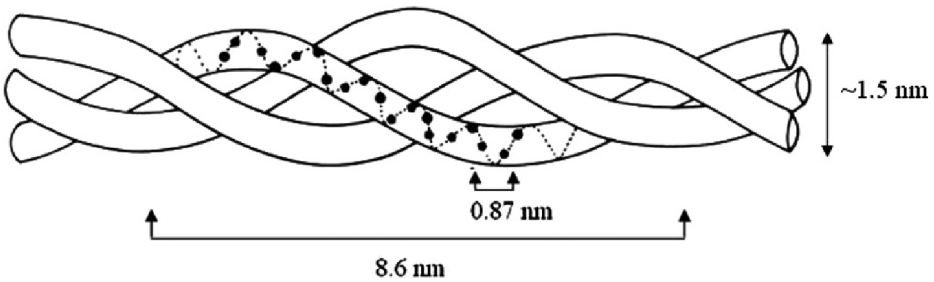


Figure 1 *Triple helix structure of collagen [8].*

Table 1 Distribution of collagen types in the body [7].

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I))_2$ α 2(I), trimmer $(\alpha1(I))_3$	Skin, tendon, bone, cornea, dentin, fibrocar-tilage, large vessels, intestine, uterus, dermis.
II	$(\alpha\ 1(II))_3$	Hyaline cartilage, nucleus pulposus.
III	$(\alpha\ 1(III))_3$	Uterine wall, dermis, skin, heart and vessels valve.
IV	$(\alpha\ 1(IV))_2$ α 2(IV)	Basement membranes.
V	α 1(V) α 2(V)(3(V) or $(\alpha1(V))_2$ α 2(V) or $(\alpha\ 1(V))_3$	Cornea, bone, large vessels, hyaline cartilage, skin,
VI	α 1(VI) α 2(VI) α 3(VI)	Skin, nucleus pulposus, heart muscle, dermis, placenta.
VII	$(\alpha\ 1(VII))_3$	Skin, placenta, lung, cartilage, cornea.
VIII	α 1(VIII) α 2(VIII)	Endothelial cells, Descemet's membrane.
IX	α 1(IX) α 2(IX) α 3(IX)	Cartilage, cornea,
X	$(\alpha\ 1(X))_3$	Hypertrophic and mineralizing cartilage.
XI	1 α 2 α 3 α1 or α 1(XI) α 2(XI) α 3(XI)	Cartilage, intervertebral disc, vitreous tumour.
XII	$(\alpha1(XII))_3$	Chicken embryo tendon, bovine periodontal ligament.
XIII	Unknown	Cetal skin, bone, intestinal mucosa.

Collagen has four levels of structural organization (Figure 2): primary, secondary, tertiary and quaternary.

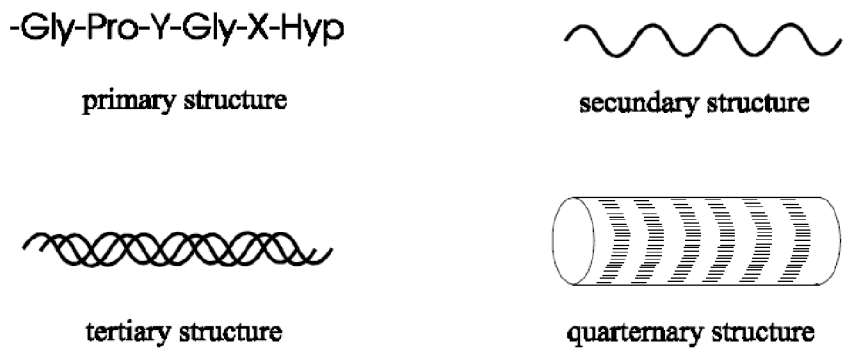


Figure 2 *The molecular architecture of the fiber forming collagens [9].*

Primary structure refers to number and sequence of amino acids. Glycine has the smallest side group and its repetition at every third position on the sequence allows close package of the chains into the helix which leaves little space for residues in the core. About 35 % of the non-glycine positions in the repeating unit Gly-X-Y are occupied by proline, found almost exclusively in the X-position, and 4-hydroxyproline, predominantly in the Y-position (Figure 3) [10].

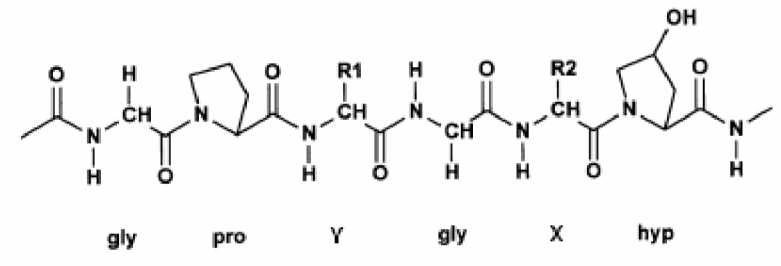


Figure 3 *Primary amino acid sequence [11].*

Secondary structure describes local structure including specific backbone torsion angles stabilized by hydrogen bonds. Tertiary structure refers to the complete three-dimensional structure – i.e. three polypeptide chains twisted to triple helical rod-like molecule so called tropocollagen. Beside hydrogen bonds, this structure is stabilized by Van der Waals bonds, ion bonds and covalent bonds as well. Covalent bond stabilization is made possible by reaction between cysteine residues in which sulphur atoms are bonded together by the removal of two hydrogen atoms. Tropocollagen molecules form high organized fibrilar structures in extracellular space. This formation is described as quaternary structure of collagen and it is stabilized by the same non-covalent forces as the tertiary structure [12].

2.1.1.1 Determination of changes in collagen structure

Infrared spectroscopy is one of the earliest experimental methods recognized as potentially useful for estimating the secondary structure of polypeptides and proteins. It has been used to study collagen cross-linking, denaturation, thermal self assembly as well as gelatin melting. The spectral changes which are indicative of changes in collagen secondary structure have been shown to include changes in the amide A (3000– 3700 cm^{-1}), amide I (1636– 1661 cm^{-1}), amide II (1549–1558 cm^{-1}) and the amide III (1200-1300 cm^{-1}) regions. It has been found that denaturation of collagen lead to reduction in the intensity of amide A, I, II and III peaks, narrowing of amide I band, increase in amide I component found around 1630 cm^{-1} and reduction in the intensity of amide I component, found around 1660 cm^{-1} [13].

2.1.2 Natural cross-links

The systematic packaging of the triple-helices provides strength and resilience to the collagen fibers. Additional mechanical and chemical stability derives from intra- and

intermolecular cross-links. Initially, the formation of cross-links is mediated by lysyl oxidase during fibril formation. 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 4a). While the fibrils associate, the aldehydes can spontaneously react. Intra-molecular cross-links are formed between two α-chains in the non-helical section of the same molecule by aldol condensation of two aldehydes (Figure 4b) [14, 15]. Inter-molecular cross-links 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) between aldehyde residues and ε-amino groups presented by lysine and hydroxylysine (Figure 4c) [15]. The inter-chain bifunctional cross-links are still reactive and continue to form polyfunctional cross-links through multiple condensations with histidine, lysine, or hydroxylysine residues. Two major products originate from reactions of aldol condensation products with histidine residues and from condensation of dehydrodihydroxylysinonorleucine with hydroxylysine (Figure 4d, e). Besides the formation of enzymatic cross-links there is a group of cross-links derived from glycated lysine and hydroxylysine residues which occur more sporadically and are important to pathobiological processes. The degree of cross-linking increases with age and stress and consequently changes the properties of collagen material. The density of cross-linking is responsible for difficulties encountered in dissolving collagen under mild conditions [7].

2.1.3 Cross-linking

Natural cross-linking gives collagen special properties, namely higher rigidity and endurance against proteolytical cleavage. However, during processing and utilization, the collagen loses those particular properties. That is why the collagenous material is additionally cross-linked by chemical or physical methods in order to regenerate original net behaviors.

The predominant chemical agents for the treatment of collagenous tissue for bioprotheses are glutaraldehyde, formaldehyde, polyepoxy compounds, acyl azide, carbodiimides and hexamethylene diisocyanate [16].

Typical physical processes are drying, heating, freezing and irradiation. The primary advantage of physical methods is that no chemicals, which may cause potential harm, are introduced so there are no residues in this approach [16].

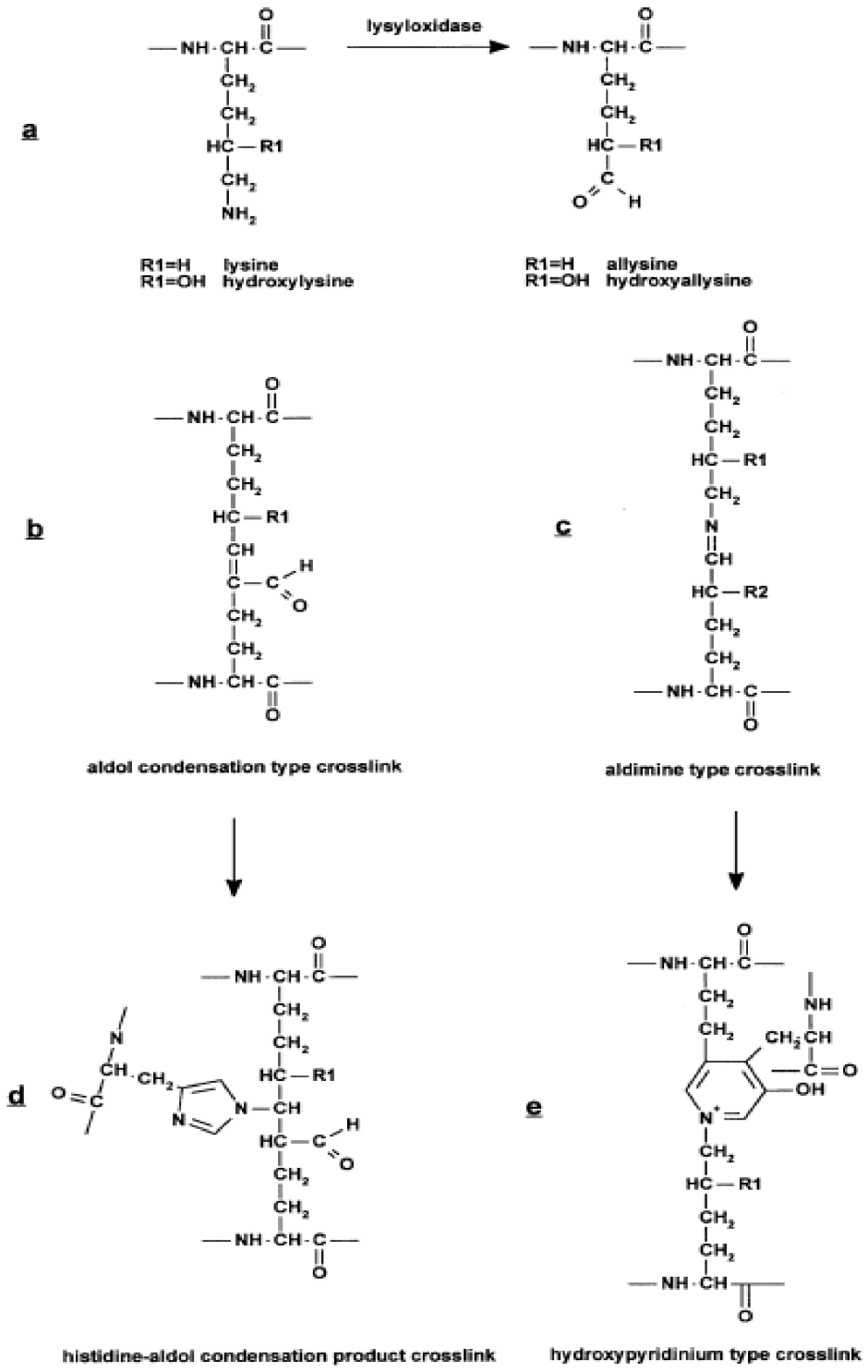


Figure 4 *Chemical structures of natural collagen cross-links, (a) Oxidation of lysine and hydroxylysine via lysyloxidase. (b) Intra-molecular aldol condensation type cross-link. (c) Inter-molecular aldimine (Schiffs' base) type cross-link, (d) condensation of aldol condensation type cross-link with hydroxyproline and (e) hydroxypyridinium type cross-link [7].*

2.1.3.1 Formaldehyde

In the 1960s, formaldehyde, a common preservative for biological tissue, was also considered together with glutaraldehyde as a candidate fixative. However, the long-term durability was found to be lower than glutaraldehyde. This was attributed to unstable and reversible fixation by formaldehyde. This is not surprising considering the single functionality of this reagent, which only permits reaction with a single collagen molecule (Figure 5). Formaldehyde reacts with the ε-amino groups of lysine and hydroxylysine residues to an intermediate imine which forms a cross-link. Formaldehyde treatment results in stretching and deformations, extreme reduction of the collagen matrix and increased immunological responses. A major advantage of formaldehyde is the possibility of cross-linking dry collagen with reagent in the vapor phase instead of treatment in liquid, in particular aqueous environments [5, 7, 16].

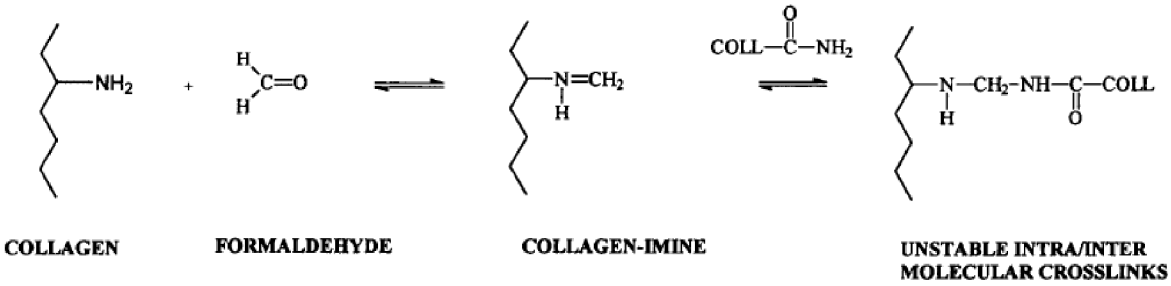


Figure 5 *Formaldehyde reactions with amino groups on collagen to form cross-links [16].*

2.1.3.2 Glutaraldehyde

Glutaraldehyde has been used as a cross-linking agent more frequently than any other reagent, since it is less expensive, readily available and highly soluble in aqueous solution. Aqueous solutions of the aldehyde consist of mixtures of free aldehyde, mono- and dihydrated monomeric glutaraldehyde, monomeric and polymeric cyclic hemiacetals and various α,β- unsaturated polymers (Figure 6) [17].

Glutaraldehyde was first successfully applied for bioprostheses in the late 1960s. Since then, many variations and conditions have been applied to optimize its efficiency. However, the specific chemistry of the glutaraldehyde fixation of collagen is not fully understood.

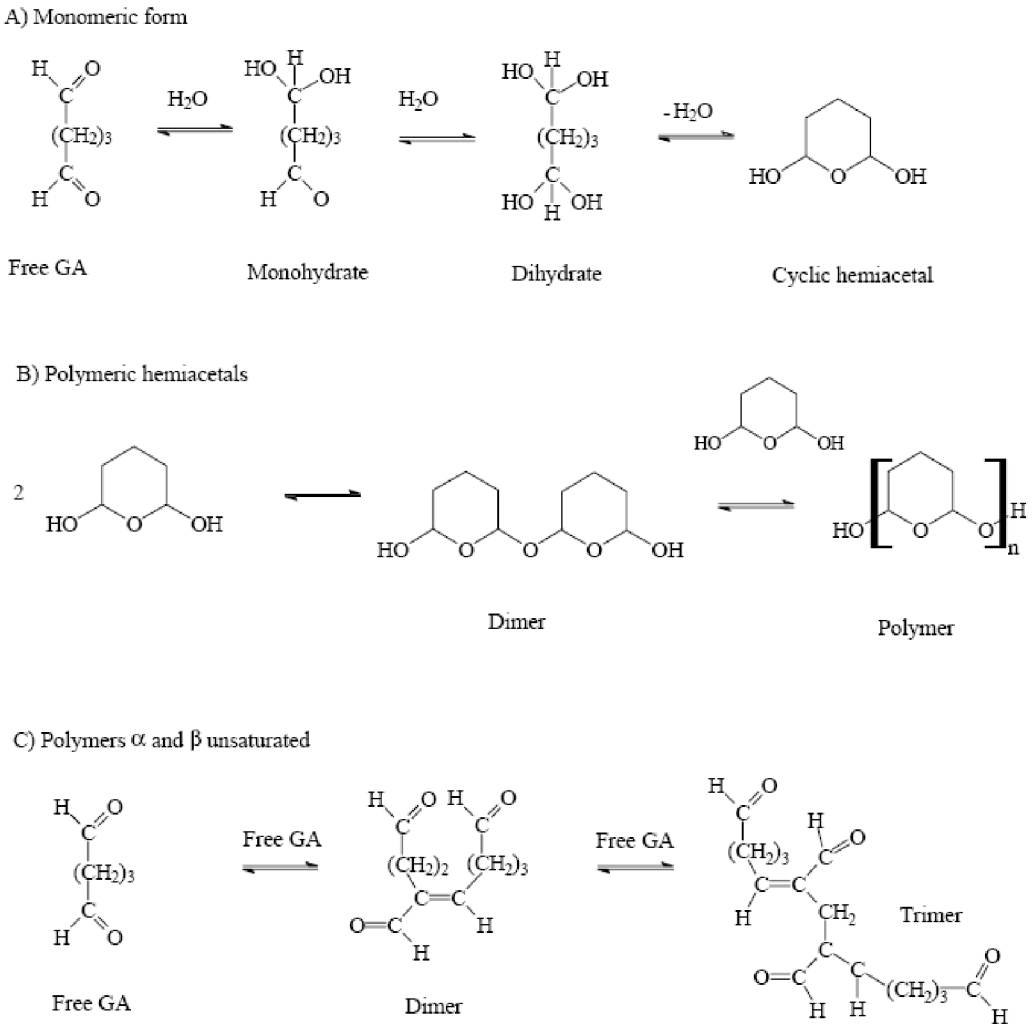


Figure 6 *Glutaraldehyde and the forms it takes in aqueous media [16].*

It is presumed that cross-linking on inter- and intra-molecular level is provided by the formation of covalent bonds. This can occur in two ways: formation of Schiff bases by reaction of an aldehyde group with an amino group of lysine or hydroxylysine (Figure 7) or an aldol condensation between two adjacent aldehydes. The Schiff base linkage is not a very stable bond, whereas the aldol condensation product is stable. Glutaraldehyde does not only interact with amino groups, but can also react with carboxy, amido and other groups of proteins [16, 17].

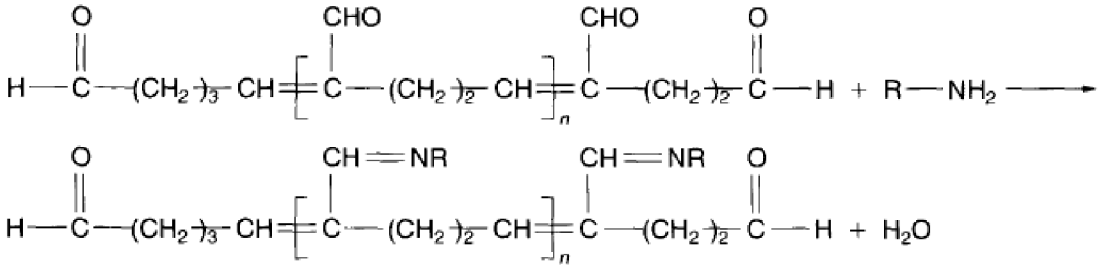


Figure 7 *Reaction of polymerized glutaraldehyde with primary amines [16].*

It was proved that the conjugated aldehyde species in the polymer (but not in the monomer) give rise to stable reaction products, whereas monoglutaraldehyde derivatives (without unsaturated groups) yield hydrolysable labile entities. Polymerization of glutaraldehyde takes place quickly, especially under alkaline pH conditions, but even at pH 5, aqueous solutions of glutaraldehyde contain polymeric species. Consequently, aqueous glutaraldehyde reacts with the amino group as an unsaturated polymer to yield a stabilized imino bond (Schiff base) [16, 17]. The order of reactivity of glutaraldehyde with certain compounds is as follows: primary amines > peptides > guanidines > secondary amines > hydroxyl groups. Compared to formaldehyde and other aldehydes, the cross-links are more stable when glutaraldehyde reacts with proteins such as albumin and collagen and mucopolysaccharides including heparin [17]. Disadvantage of using glutaraldehyde is the cytotoxicity, even in relatively low concentration. Glutaraldehyde processed bioprostheses are known to be subject to calcification in the long term, which relates to the amount of glutaraldehyde introduced into the collagen [18].

2.1.3.3 Epoxy compounds

One of the more promising approaches is the use of polyepoxy compounds (**Figure 8**) [16]. This reagent has been applied to a variety of tissues including bovine pericardium, artery and porcine aortic valve cusps and tendons compounds.

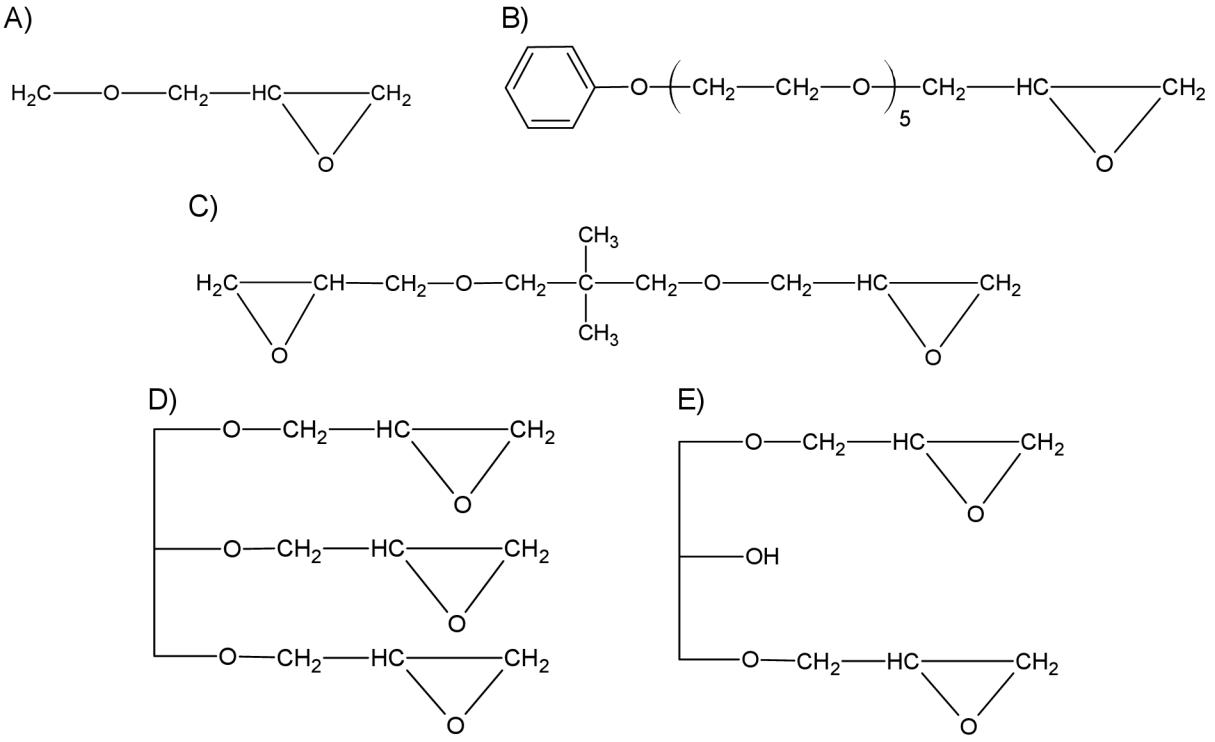


Figure 8 *Examples of chemical structures of the multifunctional and polyfunctional epoxy compounds. A) Methyl glycidyl ether, B) Phenol $(EO)_5$ glycidyl ether, C) Neopentyl glycol diglycidyl ether. D) and E) Glycerol di- and triglycidyl ether [19].*

The epoxy functional group predominantly reacts with the amino group on lysine, much like than glutaraldehyde. Figure 9 shows the proposed mechanism of cross-linking reactions with lysine in collagen. The amino group on lysine acts as the nucleophile, substituting the oxygen bond on the terminal carbon to give a carbon-nitrogen bond stable to acid hydrolysis.

Tissue cross-linked by this method has been shown to mitigate calcification and be less cytotoxic compared to glutaraldehyde-treated tissue [16].

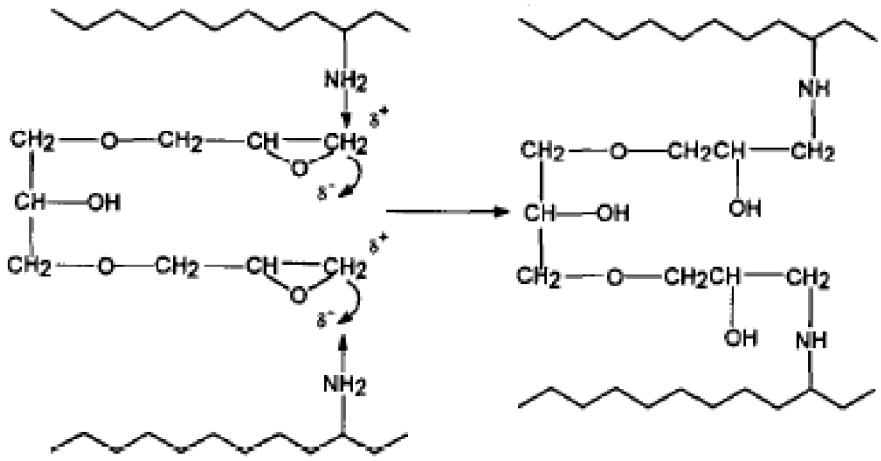


Figure 9 *Proposed mechanism of cross-linking for polyepoxy compounds to collagen [16].*

2.1.3.4 Acyl azide

In this method of the chemical fixation of collagen, a traditional bifunctional cross-linking agent is not used. Instead, the carboxyl functional groups of aspartic and glutamic acids of collagen undergo a three-step chemical reaction, and at the end, they are converted to acyl azide functionalities (Figure 10). First the carboxyl group is esterified, and then it reacts with hydrazine to form a hydrazide. Finally, the hydrazide reacts with sodium nitride to give the acyl azide. These acyl azide functionalities in turn react and couple with adjacent amino groups of other amino acids in collagen to give the cross-linked tissue. Tissue cross-linked by this method was found to have a shrinkage temperature slightly lower than the one of glutaraldehyde – fixed tissue [16].

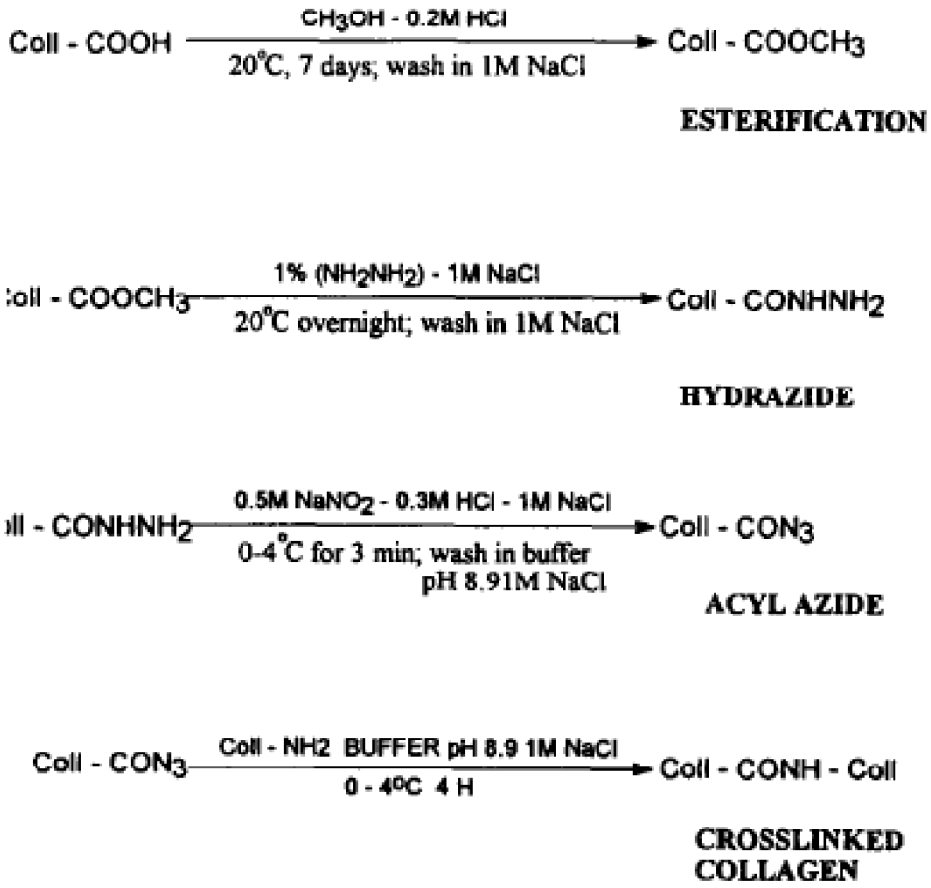


Figure 10 *The sequence of reactions of the acyl azide method to cross-link tissue [16].*

2.1.3.5 Carbodiimide [1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC)].

The carbodiimide reagent offers a method for generating cross-links between carboxylic acid and amino groups, without being itself incorporated. The water-soluble carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) is often used for cross-linking collagen (Figure 11). EDC cross-linking involves the activation of the carboxyl groups of glutamic and aspartic residues by EDC to give O-acylisourea groups. In order to suppress side reactions of O-acylisourea groups, N-hydroxysuccinimide (NHS) is used to convert the O-acylisourea group into a NHS activated carboxyl group, which is very reactive towards amino groups of (hydroxy)lysine, obtaining so-called zero-length cross-links. EDC is not incorporated in the matrix but it is converted to 1-ethyl-3-(3-dimethyl-aminopropyl)-urea which has to be removed from the cross-linking system [16, 20].

The EDC/NHS cross-linked collagen samples have a low tendency to the calcification, a good biocompatibility, a higher shrinkage temperature and enzymatic resistance than glutaraldehyde cross-linked collagen [20].

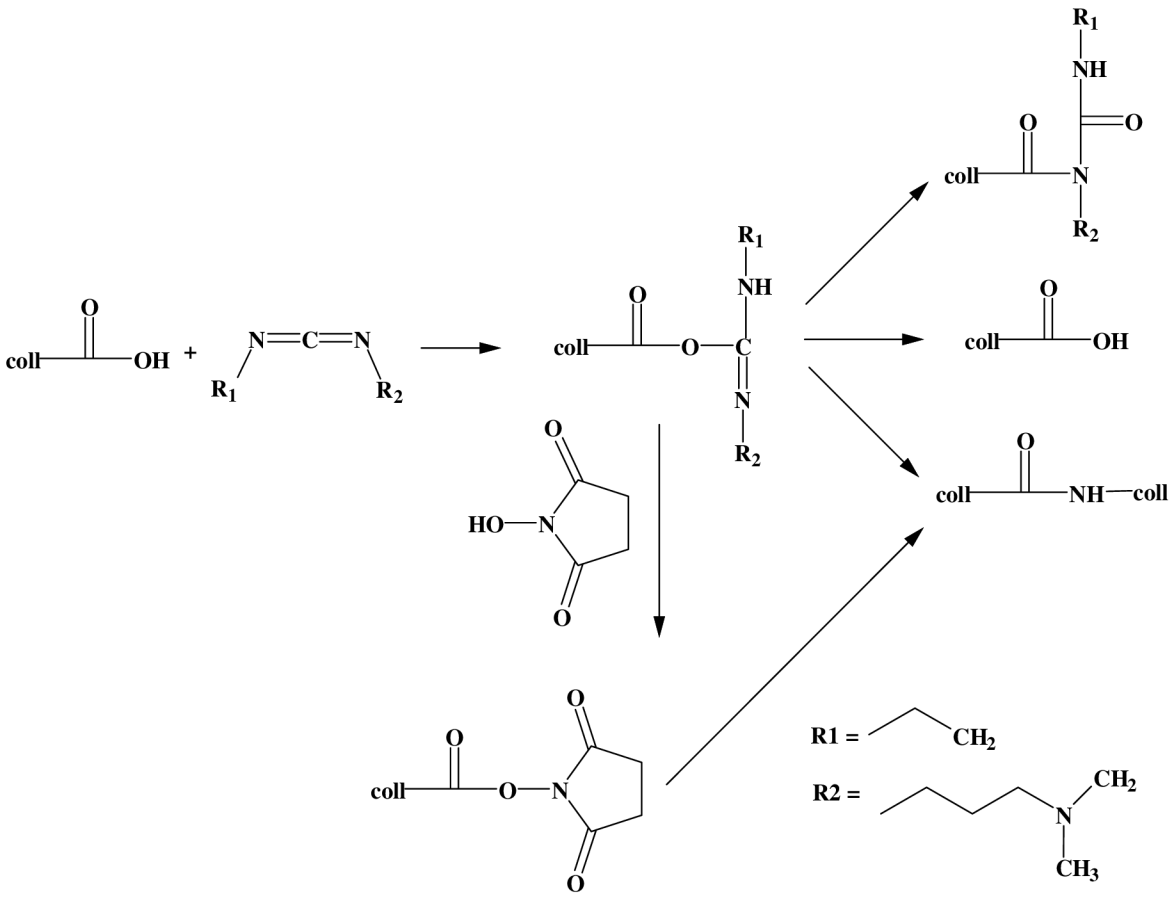


Figure 11 *Cross-linking of collagen with EDC and NHS [20].*

2.1.3.6 Hexamethylene diisocyanate

Hexamethylene diisocyanate (HMDC) is a bifunctional molecule capable of covalently bonding to amino acid residues. In the same way as glutaraldehyde, it will bond with the ε-amino groups in lysine residues and may form both intra and interhelical cross-links through urea bonds (Figure 12). The process of cross-linking is unique because it has been found that the best results are obtained when cross-linking is carried out under anhydrous conditions using 2-propanol. This is necessary to avoid the high reactivity of the isocyanate to water which destroys the isocyanate functionality before it can react with collagen [21].

HMDC-treated materials are less cytotoxic, than glutaraldehyde-treated materials. The shrinkage temperature of HMDC-treated material is comparable to glutaraldehyde-treated one [16].

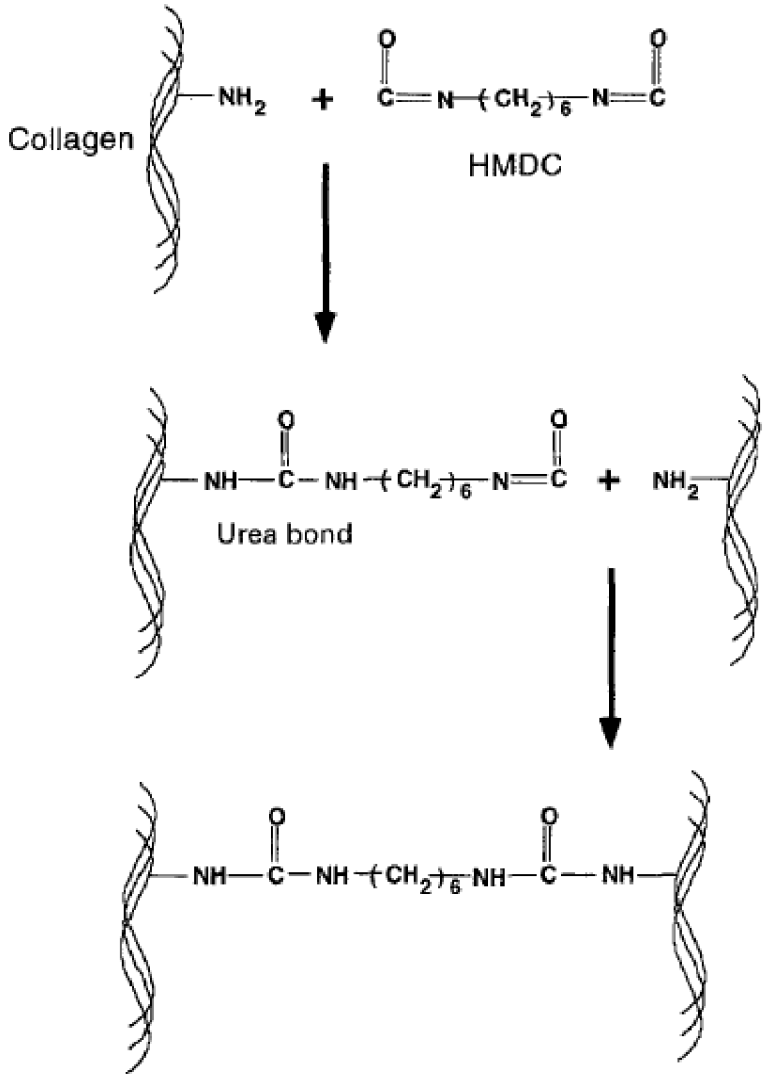


Figure 12 *Cross-linking of collagen with Hexamethylene diisocyanate [21].*

A major disadvantage of chemical cross-linking agents is the potential toxic effect of residual molecules and/or compounds formed during in vivo degradation.

2.1.3.7 Physical methods

Typical physical processes are dry heat and γ-irradiation. Both dehydrothermal treatment (DHT) and exposure to ultraviolet light at 254 nm increase the collagen shrinkage temperature. The resistance to collagenolytic degradation and the durability under load in collagenase. However, collagen becomes partially denatured by these physical treatments. In order to keep degradation of the triple-helices at the minimum level, it is crucial for DHT treatment to reduce the water content via vacuum as much as possible prior to heating. Even small amounts of residual moisture can cause breakdown of the helical structure and proteolysis. Strong dehydration itself already induces amide formation and esterification between, carboxyl and free amino groups, carboxyl and hydroxyl groups, respectively. But

the effect is insignificant and typical DHT conditions are 110 °C for several hours up to a few days. The combination of degradation and cross-linking allows non-specific enzymes to attack and solubilize fragments of the cross-linked material. Sometimes, DHT treatment is followed by a chemical treatment (cyanamide) to increase the stability of the treated material [7, 9, 22].

The mechanism of collagen stabilization under these conditions is unknown but Bello and Riese-Bello [22] suggested the formation of amide cross-links between amine and carboxyl groups. A possible alternative cross-linking route is through the formation of lysino-alanine following β-elimination of serine residues and subsequent reaction of the resultant dehydro-alanine with lysine. Such a mechanism could lead to intermolecular cross-links in the collagen matrix which could assist in stabilizing the heat-cured material [21].

Formation of cross-links during UV-irradiation is thought to be initiated by free radicals formed at aromatic amino acid residues which indicate a rather limited maximum degree of cross-linking due to the small number of tyrosine and phenylalanine residues in collagen. Exposure time can therefore be kept short because cross-linking density reaches its limits soon. Nevertheless, UV-irradiation improves mechanical strength [7, 24].

Finally, Moore *et al.* used a dye-mediated photo-oxidizing method to cross-link bovine pericardial tissue. This method, which led to the modification of histidine, tryptophan, tyrosine, and methionine, resulted in materials which were resistant to pepsin and cyanogen bromide (CNBr) treatment. Remarkably, the shrinkage temperature was similar to the untreated material, which suggests that the tissue behaves like the original and that the cross-links did not influence the tissue character [9].

2.1.4 Determination of free amino groups

For the investigation of cross-linking degree the UV-VIS methods to determinate free amino groups are mainly used. In this work, evaluation of the amount of free amino groups in collagen samples took place throughout using two reagents - ninhydrin and 2,4,6-trinitrobenzenesulfonic acid, which reactions are briefly described bellow.

2.1.4.1 Ninhydrin reaction

Redox reaction of ninhydrin (2,2-dihydroxy-1.3-andanione) with free amino and imino groups gives colored product. Primary amino groups react with ninhydrin reagent to form the violet product of diketohydrindylidene-diketohydrindamine (λ_{max}= 570nm) (Figure 13). Imino groups give a yellow product (λ_{max}= 440nm). In peptides and proteins, where the amino acids are linked to the peptide bonds, the ninhydrin react only with free ε-amino groups of lysine [25, 26].

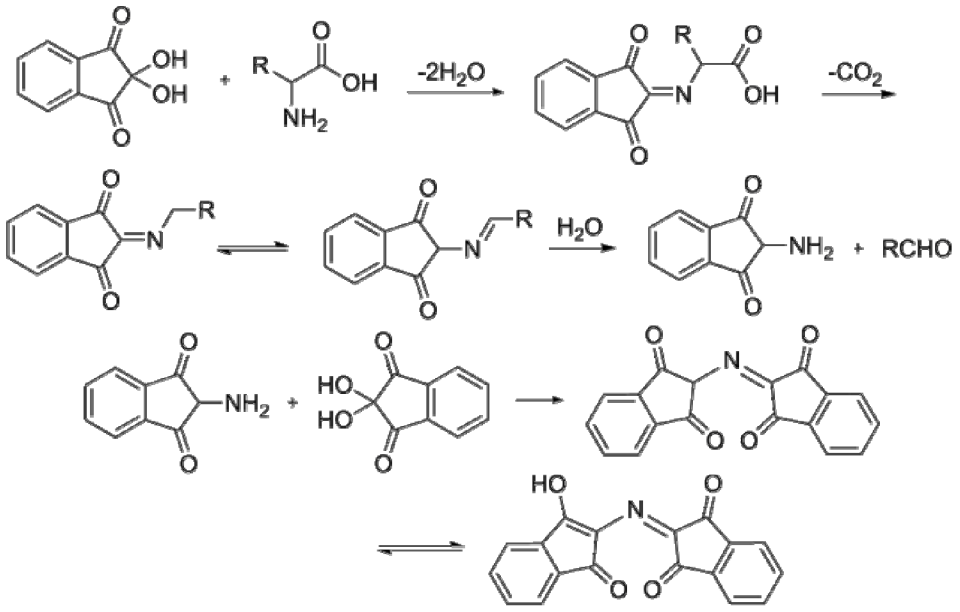


Figure 13 *The mechanism of the ninhydrin reaction [27].*

2.1.4.2 2,4,6-trinitrobenzenesulfonic acid (TNBS)

A useful reagent of 2,4,6-trinitrobenzenesulfonic acid (TNBS) was developed by Okuyama and Satake (28) and Satake et al. (29) to determine the free amino groups of amino acids and peptides in column eluates. The reagent was found to react specifically and under mild conditions with the free amino groups to give trinitrophenyl (TNP) derivatives [30]. Firstly, the reaction would begin with a nucleophilic attack of the non protonated amino group (pK next to 10) on an aromatic carbon and would lead to an addition–elimination. This aromatic nucleophilic substitution gets through the Mesenheimer salt, which is a very stable complex (Figure 14) despite the leaving group of SO_3^{2-} [31].

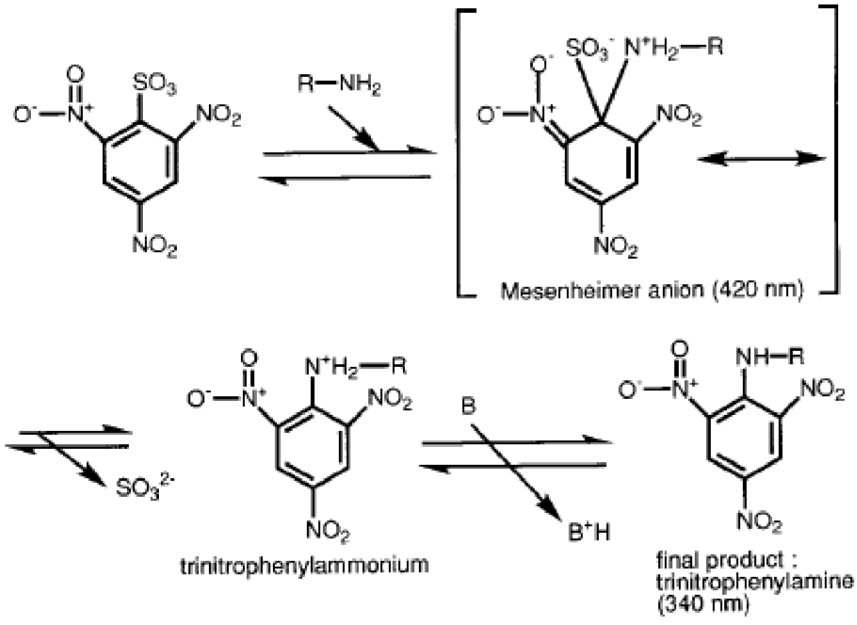
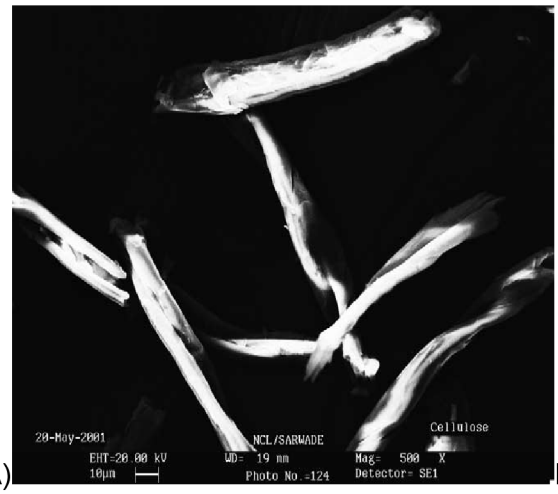


Figure 14 *The mechanism of the TNBS reactions [31].*

2.2 Oxidized Cellulose

Cellulose (Figure 15 A) is the most abundant renewable biopolymer in nature and it has the potential to become a key resource in the development of sustainable biofuels and biomaterials [32]. As a biomaterial, cellulose can be converted into a wide range of derivatives with desired properties for a variety of biomedical applications. The three hydroxyl groups of the cellulose molecule can undergo chemical reactions common to all primary and secondary alcohol groups, such as etherification, nitration etherification, and oxidation. From these reactions, a variety of useful polymers can be created [33].

A) 

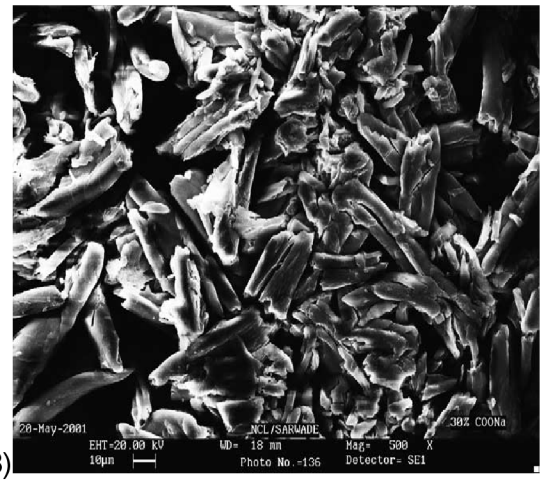
B) 

Figure 15 *Image of the cellulose fibres that have an aspect ration 14 A) and image of 30 % oxidized cellulose B). It is obvious from images that during the oxidation, the reduction of fibers length occurs [34].*

2.2.1 Cellulose oxidation

Oxidation, however, is the only process that renders cellulose bio-absorbable in human. A major problem with oxidation is the difficulty of producing materials that are homogeneous in chemical and physical properties. This complication varies from the different reactivities of the three hydroxyl groups, the similar availabilities of different parts of the cellulose molecule and the distinct behavior of different oxidants [33].

Nitrogen dioxide is one of the most selective oxidants that convert primary hydroxyl cellulose groups into carboxyls (Figure 16). The formation of side products depends mostly on oxidation conditions. Oxidized cellulose can be prepared in a reaction of cellulose with liquid or gaseous nitrogen dioxide or in a solution with different organic solvents. The oxidation of cellulose in liquid N_2O_4 is rather slow and yields a product with a high content of side (nitroester and carbonyl) groups. Oxidation in a gaseous state at a high temperature (70 °C) and pressure (7 MPa) leads to obtain in a short period of time. Oxidized cellulose with the maximum possible carboxyl content [35].

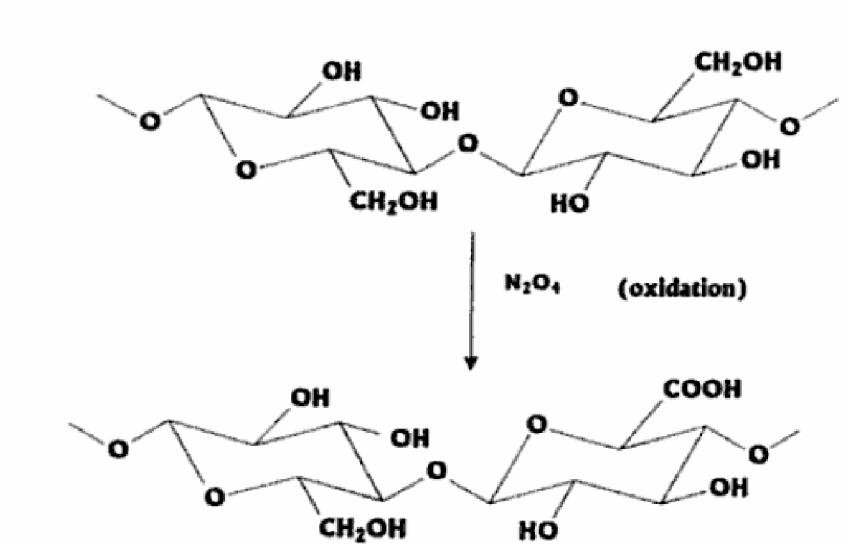


Figure 16 *Chemical structure of oxidized cellulose [33].*

2.2.2 Determination of free carboxyl groups

Currently, an oxidized cellulose (Figure 15B) containing 16–24% carboxylic group is commercially available in powder gauze and fabric forms. To quantitatively determine the carboxyl content in oxidized cellulose several methods have been developed. These include: an alkalimetric titration, a calcium exchange method, an iodometric titration method, CO_2 evolution method and a methylene blue adsorption method. However all these methods are destructive and have limitations. For example the alkali metric method is good only if no carbonyl groups are present. The calcium exchange method on the other hand requires steeping of the sample with an acid followed by extensive washing with water prior to analysis. During steeping some of the carboxyl groups may convert into lactones and consequently may result in lower carboxylic content values. On the other site for determination of carboxyl content in oxidized celluloses a noninvasive method solid-state carbon-13 cross-polarization–magic angle spinning nuclear magnetic resonance (13C CP MAS NMR) spectroscopy can be used. This method is simple and requires no sample preparation [36].

2.2.3 Application

Oxidized celluloses represent an important class of biocompatible and bio-absorbable polymers. Oxidized cellulose is commercially available in a sterilized knitted fabric or powder form for use in humans to stop bleeding during surgery and to prevent the formation of inflammations following surgery. These materials have been widely used in medicine for several decades. Studies show that oxidized cellulose with at least 3% of carboxylic content is bio-absorbable. Recently oxidized cellulose has also been found to possess antibacterial, antitumor, immunostimulant and wound healing properties. This beneficial effect is immediate and is induced by a low pH effect. The current theory is that this chemical haemostatic reduces the effective initial inoculums with an acid hostile ambient allowing the

host's natural defenses to overcome the organism. Owing to the presence of carboxylic groups oxidized cellulose has also been extensively investigated as an immobilizing matrix for a variety of amine drugs for enzymes and for bovine serum albumin [37, 38].

Oxidized cellulose is one of the fastest-degrading and resorbing known polymers. When oxidized cellulose is dissolved in neutral pH water, no appreciable hydrolysis occurs. If the pH of water is increased, the structure breaks down. Oxidized cellulose disappears completely in 21 days when placed in phosphate-buffered saline (PBS). Similarly, it dissolves 80 percent after 2 weeks in vivo. It is classified as biodegradable since it degrades without the help of enzymes [33, 39].

Because the oxidized cellulose contains the carboxyl groups it can be used for cross-linking and stabilization of collagen and thus replace the toxic chemical cross-linking agent. In order to increase the reaction efficiency, the carboxyl groups of oxidized cellulose could be activated using the EDC/NHS system.

3 EXPERIMENTAL PART

3.1 Chemicals

- Collagen - Type I collagen, 8% aqueous solution, Výzkumný ústav pletařský. a.s., Czech Republic.
- Oxidized cellulose – 0,6 % of carboxyl groups' content, Synthesia, a.s., Czech Republic.
- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) - Sigma Aldrich Chemical Company, Germany.
- *N*-hydroxysuccinimide (NHS) - Sigma Aldrich Chemical Company, Germany.
- 2.2-Dihydroxy-1.3-indanedione (Ninhydrin) - Sigma Aldrich Chemical Company. Germany.
- 2,4,6-Trinitrobenzenesulfonic acid (TNBS) - 5% solution, Sigma Aldrich Chemical Company, Germany.
- ETHANOL – 98%, Lach-Ner, s.r.o., Czech Republic.
- MILIQ VODA – prepared by Millipore S.A.S at FCH BUT.
- Lysin – Sigma Aldrich Chemical Company, Germany.
- Sodium dodecyl sulfate – 10 %, Penta, Czech Republic.
- Hydrochloric acid –1 M, Lach-Ner, s.r.o., Czech Republic.
- $Na_2HPO_4.12\ H_2O$ – 0.1M water solution, Lach-Ner, s.r.o., Czech Republic.
- Calcium acetate – 2% water solution, Lach-Ner, s.r.o., Czech Republic.

3.2 Instrumentation

- Analytical balance Mettler Toledo classic, type AB204S
- FT-IR spectrometer Nicolet iso10
- Desintegrator IKA Turrax basic T18. D
- Centrifuge Hettich EBA 30. D
- Lyophilizator LABCONCO, Freeze Dry System/Freezone 4.5
- Scanning electron microscope FEI Quanta 200 Mk2
- GPC chromatograph Agilent 1100 Series
- UV-VIS Spectrophotometer Helios Alpha Unicam
- CO incubator Sanyo model MCO – 18AIC

3.3 Sample preparation

3.3.1 Preparation of collagen/oxidized cellulose polymer mixtures

Eight different collagen/cellulose mixtures were prepared (see Table 2) from lyophilized collagen and oxidized cellulose powder.

Table 2 *Composition of the sample*

Ratio Collagen/cellulose	Weight [g] collagen/cellulose	Volume of mixtures [ml]
1:0	0.075 : 0.0000	25
9:1	0.075 : 0.0083	25
3:1	0.075 : 0.0250	25
5:3	0.075 : 0.0450	25
1:1	0.075 : 0.0750	25
1:2	0.075 : 0.1500	25
1:3	0.075 : 0.2250	25
1:9	0.075 : 0.6750	25

Firstly, the collagen water solution (3 $mg.ml^{-1}$) was prepared by disintegrating collagen fibers using IKA disintegrator at 8000 rpm in miliQ water. Consequently, the calculated cellulose powder for each weight ratio was dissolved in 5 ml of miliQ water by heating at 40 ºC. The homogeneous cellulose solution was added to the prepared collagen solution and the polymer mixture was stirred by disintegrator another three minutes to homogeneous state.

3.3.2 SDS collagen/cellulose mixture modification

Due to the phase separation of some collagen/cellulose mixtures (Figure 17) 1ml of sodium dodecyl sulfate (SDS) was added to the collagen solution during the homogenization. Consequently the cellulose solution was added and mixed. These polymer mixtures were used for air-dried film preparation and determined the free amino groups.

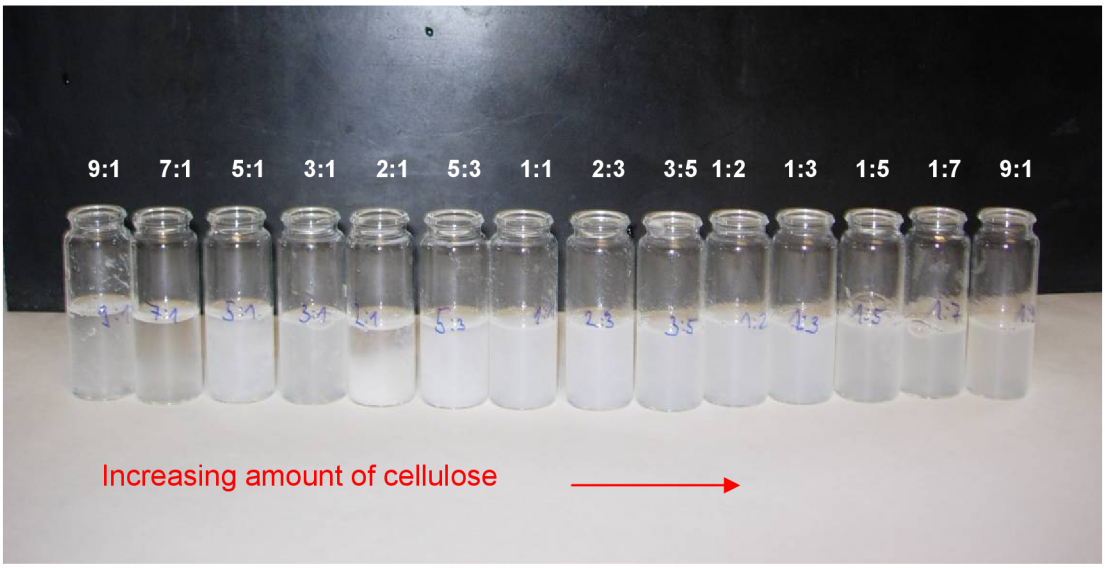


Figure 17 *Prepared collagen/cellulose mixtures.*

3.3.3 Preparation of collagen/cellulose films

The 10 ml mixtures of collagen/cellulose were poured on Petri dishes with a diameter of 5 cm and were air-dried for 24 hours until constant weight.

3.3.4 Preparation of collagen/oxidized cellulose lyophilized sponges

The 10 ml mixtures of collagen/cellulose were poured on Petri dishes with a diameter of 5 cm and were frozen quickly by liquid nitrogen at -196 °C or slowly in the freezer at -30 ºC. The frozen samples were lyophilized (freeze-dried) at -80 ºC and 15 Pa for 24 hours.

3.3.5 Activation of cellulose carboxyl groups using EDC/NHS

The EDC/NHS is used with the aim of activating cellulose carboxyl groups and accelerates reaction of cellulose carboxyl groups with collagen amino groups. The expected reaction mechanism of cellulose carboxylic group activation and their subsequent reaction with the amino group of collagen to produce amide bond is shown in the Figure 18.

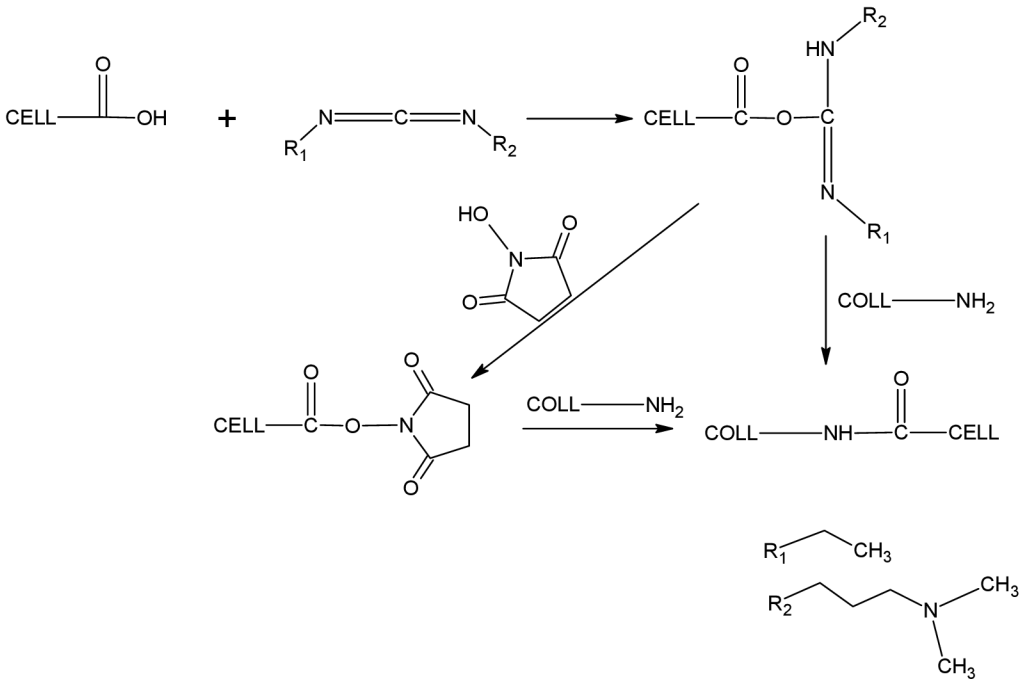


Figure 18 *Activation of carboxyl groups using EDC/NHS.*

3.3.5.1 Activation in situ

The 25 ml polymer blends were modified using ethanol solution of 50 mmol.l^{-1} EDC and 25 mmol.l^{-1} NHS in situ. The carboxyl groups of cellulose were activated in the solution for 4 hours at room temperature. Consequently, this solution was added to the collagen solution and mixture was homogenized, poured in Petri dish and dried. Prepared film was washed out with 20 ml of 0.1 mol. l^{-1} Na_2HPO_4 for 2 h followed by 20 ml of miliQ water for next 2 h.

3.3.5.2 Activation in the form of film

The film, which was prepared from the 25ml polymer mixture of collagen/cellulose, was modified in the Petri dish for 4 hours by the same concentration of EDC/NHS solution like in previous samples. Consequently, the activated films were carefully washed out with 20 ml of 0.1 mol. l^{-1} Na_2HPO_4 for 2 h followed by 20 ml of miliQ water for next 2 h.

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3.4 Characterization of the samples

3.4.1 *Determination of* free *amino groups*

For the determination of free amino groups in collagenous samples two reagents - ninhydrin and 2,4,6-trinitrobenzenesulfonic acid were used. The resulting colored products were evaluated using UV-VIS spectroscopy. L-lysine was used as a standard for the calibration curve.

3.4.1.1 Ninhydrin reaction

The collagen/cellulose mixture (2 ml) was placed into 20 ml vial. Then 1 ml of 0.2 % ethanol solution of the ninhydrin was added. The solution was stirred and simultaneously heated up at 100 ºC for 20 min. Subsequently, the solution was three times diluted (9 ml of miliQ water) and then measured by UV-VIS at 570 nm in a 1 cm cell against blank, which consisted water instead of the sample. For each ratio five samples were prepared and measured.

Percentages of free amino groups in the sample were calculated using the equation (4), where c_s is the concentration of amino groups in the sample and c_c is the concentration of amino groups of lysine in the pure lyophilized collagen.

$$\text{Free } NH_2 \text{ groups } (\%) = \frac{c_s}{c_c}.100 \quad (4)$$

3.4.1.2 2,4,6-trinitrobenzenesulfonic acid (TNBS)

To 2 ml of the collagen/cellulose mixture 1 ml of 0.01 % TNBS and 2 ml of 0.1M $NaHCO_3$ for the pH adjustment to 8.5 were added. The solution was allowed to react at 40°C for 2 hours and then 1 ml of 10% SDS was added to solubilize the protein and prevent its precipitation by addition of 0.5 ml 1 M HCl. The absorbance of the solution at 340 nm was measured against a blank sample treated as above but with 2 ml of water instead of the collagen/cellulose solution. The percentages of amino groups of the samples were calculated using equation (4) above.

3.4.2 Determination of carboxyl groups in oxidized cellulose

Determination of the carboxyl groups in oxidized cellulose was performed according to the method employed by Kumar and Yang [39]. About 0.5 g of oxidized cellulose powder was accurately weighed and soaked in 50 ml of 2% (w/v) calcium acetate solution for 30 min. The mixture was titrated with a 0.1M NaOH solution using phenolphthalein as an indicator. The carboxyl content in oxidized cellulose was calculated according to equation (1).

$$\text{carboxyl content } (\%\ \text{w/w}) = \frac{\text{N.V.Mw}_{\text{COOH}}}{\text{weight of sample (mg)}}\ x\,100 \quad (1)$$

Where N is the molarity of NaOH, V is the volume (ml) of NaOH consumed in titration after comparing with the blank blank and Mw is the molecular weight of the oxidized cellulose.

3.4.3 Characterization of the collagen/cellulose mixture suspension

For the investigation the amount of precipitated polymer and polymer dissolved in water, the collagen/cellulose mixtures were separated using centrifuge. The precipitated part was separated by decantation from the polymer solution and both parts were air-dried and

weighted. The percentage of precipitated or non-precipitated part was calculated using equation (3).

$$\text{Separated polymer}\,(\%) = \left(\frac{\text{weight of the polymer after separation (g)}}{\text{original weight of total sample (g)}} \right).100 \qquad (2)$$

3.4.4 Degradation study

The samples in the form of a film were cut at the same small pieces, weighted and put into vials containing 10 ml of miliQ water. Prepared vials were placed into incubator at 37°C and every day the films were taken out, placed on Petri dishes to dry the excess water and weighted. The degradation was calculated according to equation (3), where M_P is the original sample weight and M_D is the sample weight after degradation.

$$\text{weight loss}\,(\%) = \left(\frac{M_p - M_D}{M_P}.100 \right) \qquad (3)$$

3.4.5 Gel permeation chromatography (GPC)

Number average molecular weight (Mn) and polydisperzity index (PDI) of oxidized cellulose were determined using GPC. Water with flow of 1ml.min^{-1} was used as a mobile phase and polyethylene oxide as a standard.

3.4.6 Amino acid analysis

Amino acids were determined in lyophilized collagen hydrolyzed by 6 M HCl acid for 24 hours at 110 °C. This method is not suitable for the determination of sulfur amino acids.

3.4.7 Fourier-transformed Infrared spectroscopy (FT-IR)

FT-IR analysis was carried out for the qualitative determination of the collagen denaturation on the secondary structure level. For the measurement, new films were prepared from the polymer mixtures. 5 ml of the mixtures was placed into Petri dishes and were air-dried for 24 h. Measurement of infrared spectra was made by the ATR method using ZnSe crystal, at the resolution 4 and 128 scans.

3.4.8 Morphology

For the morphology evaluation, 5ml of the polymer mixture with concentration of 3 mg.ml^{-1} was placed into Petri dishes with 5 cm diameter and frozen using liquid nitrogen or in the freezer in order to see the difference between the porosities and pore sizes of lyophilized samples. The morphology of lyophilized collagen/cellulose scaffolds was studied using scanning electron microscopy (SEM). From the obtained pictures the porosity and pore size of collagen/cellulose matrices were characterized using image analyses program ImageJ.

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4 RESULTS AND DISCUSSION

4.1 Characterization of oxidized cellulose

4.1.1 Determination of free carboxyl groups

The carboxyl groups content, which is important for cross-linking of collagen, has been determined using titration method. The results are shown in Table 3. As it is seen, the calculated amount of carboxyl content in the oxidized cellulose was found to be approx. 0.68 %. The supplier shows carboxyl content of 0.6 %, which is in very good accordance with our determined value.

Table 3 The measured and calculated value for oxidized cellulose.

Weight of sample (mg)	Volume of NaOH used for titration (ml)	Carboxyl content (hm %)
499.5	0.75	0.6758
500.4	0.80	0.7194
501.2	0.80	0.7183
500.9	0.75	0.6738
500.8	0.70	0.6290
	Average:	0.6833

4.1.2 GPC analysis

The molecular weight of oxidized cellulose was determined using GPC analysis. The chromatogram obtained from the analysis is given on the Figure 19. Mn of oxidized cellulose was about 128200 Da and PDI around 10.1 showing very broad molecular weight distribution, which is very common for natural polymers.

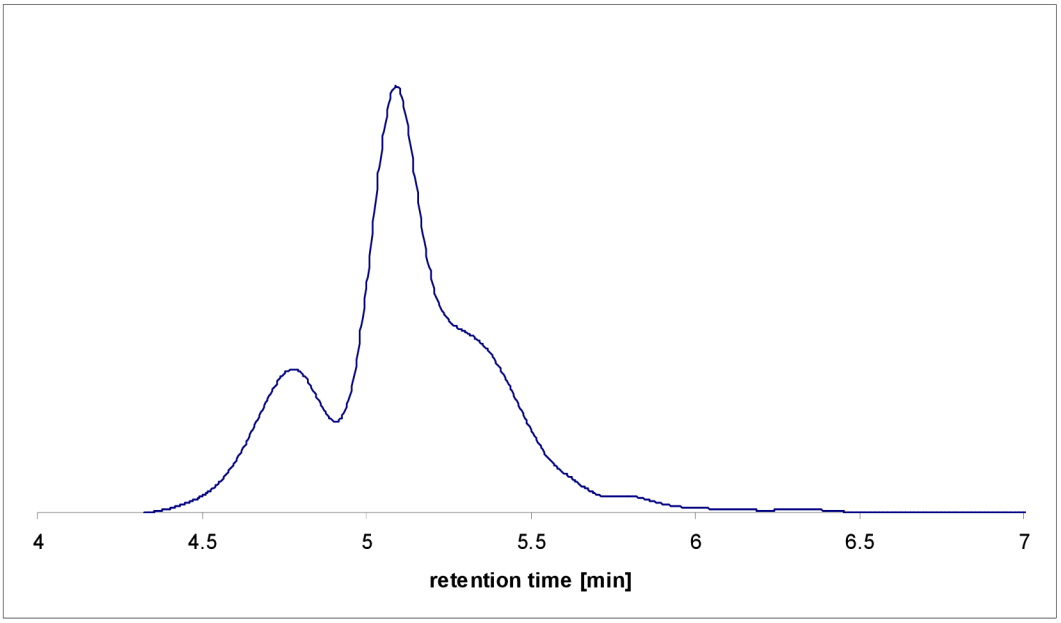

Figure 19 *Chromatogram of the oxidized cellulose from the GPC analysis.*

4.2 Amino acid analysis of collagen

Amino acid analyzer was used for the characterization of amino acid composition of collagen in collagenous samples (Table 4). The amino acid compositions directly influence the collagens physic–chemical properties (e.g., solubility in NaCl solution, cross-linking ability and thermal stability). For the cross-linking of collagen, amino acid lysine whose amino group is involved to cross-linking of collagen is important.

Table 4 Amino acid composition of the collagen

Nitrogen substance (NL)	915.1 g/kg
Asp	56.2
Thre	17.3
Ser	30.4
Glu	84.1
Pro	133.1
Gly	108.3
Ala	78.9
Val	21.7
Met	7.5
Iso	14.2
Leu	29.0
Tyr	5.9
Phe	19.5
His	9.5
Lys	33.8
Arg	90.6

4.3 Characterization of the collagen/cellulose mixture suspension

The resulting values of the precipitated and non-precipitated polymer amounts of separated polymer mixture are shown in the Table 5 and graphically in Figure 20. Because the collagen is poorly soluble in water there are two parts already in pure collagen solution (suspension) - the soluble (non-precipitated) and insoluble (precipitated) one. Both parts had the same weigh of about 50 % of original weight of sample. If the small amount of cellulose is added to collagen the mixture start agglomerate and the weight of precipitate significantly increase up to value 90 % of original weight of sample at ratio 9:1. With additional increasing amount of cellulose the amount of precipitated part decreases to the ratio 1:2. From that time the mixtures were not agglomerated and the amount of precipitated and non-precipitated part were same of about 50 % until the 1:9 ratio.

From these results it is evident that between cellulose and collagen some interactions have occurred. These interactions have probably only physical character since both collagen

and cellulose have functional carboxyl groups. There might be a ionic interactions between collagen and cellulose at the low cellulose concentrations as well. However, if there are an equilibrium between collagen and cellulose or even higher amount of cellulose than collagen, the mixtures do not agglomerate and cellulose probably acts as a solvent for the collagen with diminishing weak physical interactions.

Table 5 Resulting values of precipitated and non-precipitated part of the collagen/cellulose mixtures.

Ratio collagen/cellulose	Original weight of mixtures (g)	Precipitated polymer (wt. %)	Polymer in solution (wt. %)
1:0	0.0715	49.37	50.63
9:1	0.0695	90.07	9.93
3:1	0.0951	86.43	13.57
5:3	0.1105	74.30	25.70
1:1	0.1295	63.78	36.22
1:2	0.1978	47.42	52.58
1:3	0.2883	51.58	48.42
1:9	0.6832	49.82	50.18

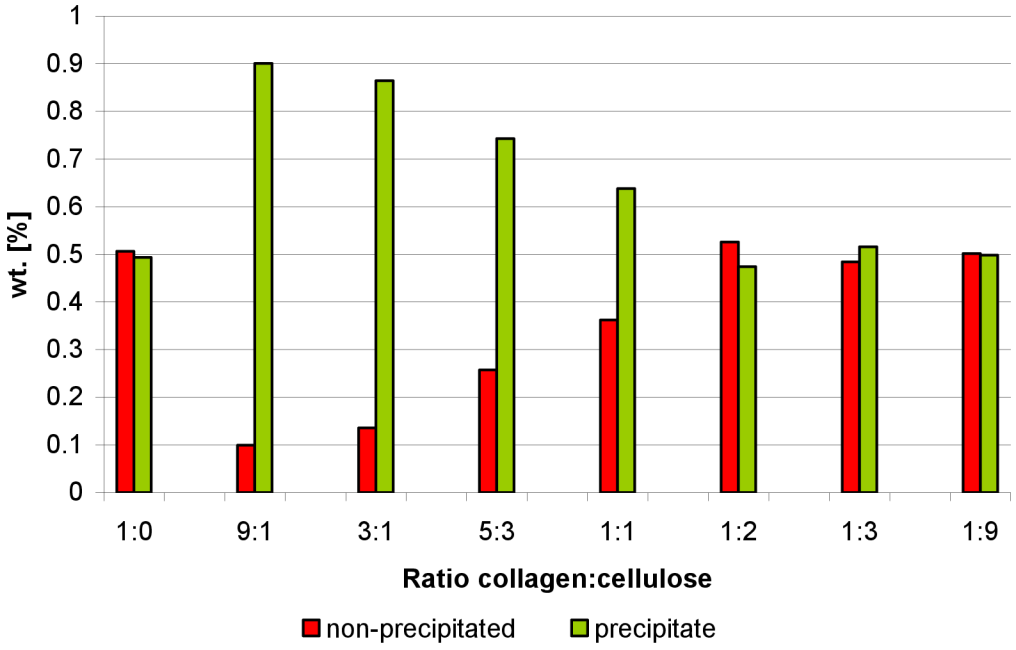

Figure 20 *Precipitated and non-precipitated parts of collagen/cellulose mixtures.*

4.4 Degradation

The dependences of weight loss on the time for unmodified samples and samples modified using EDC/NHS (50 $mmol.l^{-1}$/25 $mmol.l^{-1}$), which were unwashed during the degradation evaluation are shown in Figure 21 and Figure 23, respectively. Figure 22 and

Figure 24 .represent unmodified and modified samples, respectively, which were washed out every day with clean water. Degradations were done in incubator at 37 °C.

Based on presented graphs, it can be seen, that pure collagen slowly degraded with increasing time of degradation and after 10 days reaches loss weight of 66 % for unmodified and unwashed sample and 52 % for unmodified and washed sample. If the cellulose was added the polymers were precipitated, the loss weight decreased and in the ratio 9:1 the loss weights reach value 28% and 20 % for the unmodified and washed samples, respectively. This behavior continues up to ratio of 1.5:1, which from the loss weight increase and at the ratio of 1:1 the precipitation was ceased. At the ratio of 1:3 almost same values as the pure collagen had were reached, approx. 62 % and 64 % for unmodified and washed samples, respectively. It is evident that after small addition of cellulose to collagen the stability of polymer mixture increases. This can be explain by the creation of physical interactions between cellulose and collagen and beyond the equilibrium state cellulose works more like solvent, which is confirmed by increasing loss weight that start at ratio of 1.5:1 collagen/cellulose.

The obtained results of samples which were washed out within a degradation study are very similar with the value of unwashed samples. It can be concluded that washing the sample during the degradation of the unmodified samples does not have any effect.

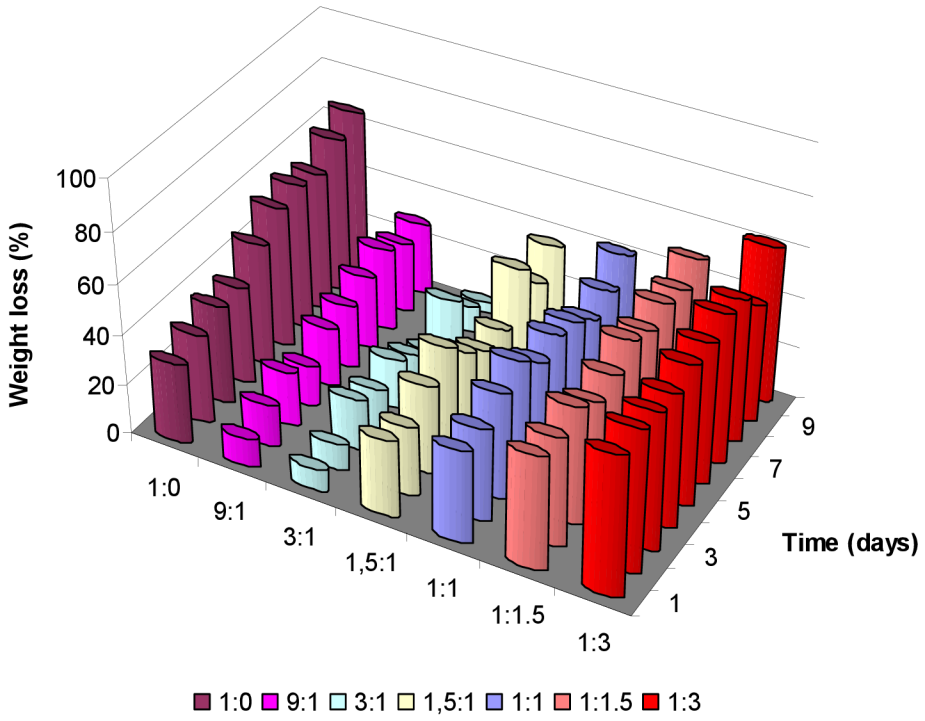

Figure 21 *The dependence of the weight loss on the degradation time for unmodified and unwashed collagen/cellulose mixture.*

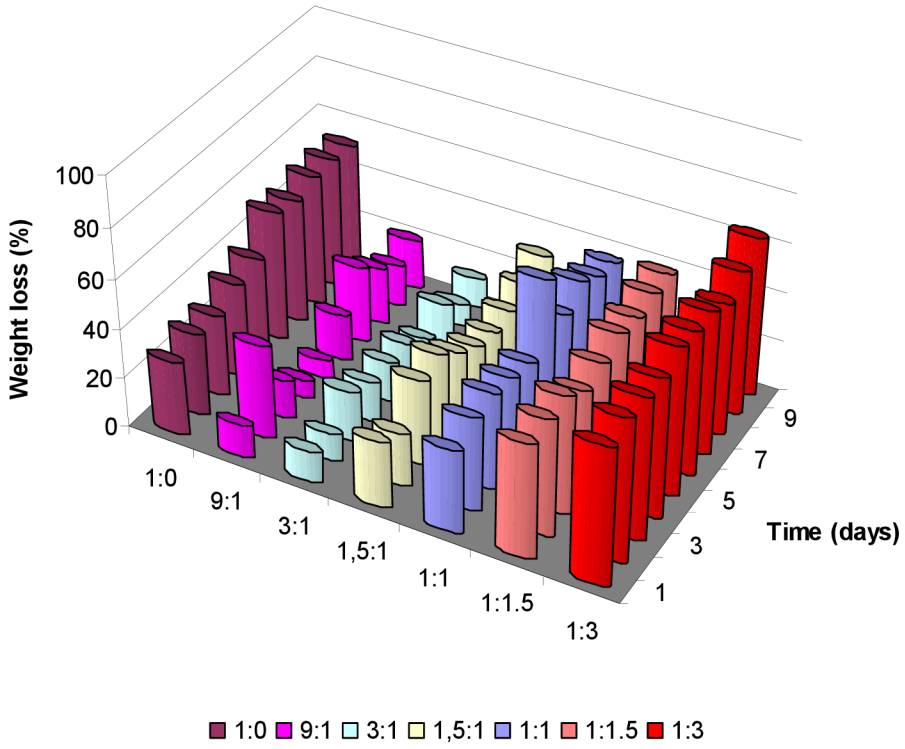

Figure 22 *The dependence of the weight loss on the degradation time for unmodified and washed collagen/cellulose mixture.*

If the sample is cross-linked the stability against the degradation is higher. Pure collagen samples modified with EDC/NHS and unwashed lost after 10 days only 14 % from the original weight. If the samples were washed the weight loss was higher (about 36 %). This can be due to cross-linking of samples with excess of EDC/NHS in the unwashed samples. Since the sample is washed out during the degradation the cross-linking agent is removed from the mixture and collagen can not be additionally cross-linked. If the cellulose is added to collagen solution the behavior is comparable with the unmodified samples. Thus it is evident that EDC/NHS system did not activate the carboxyl groups of oxidized cellulose to create stabilizing amide bond with amino groups of collagen.

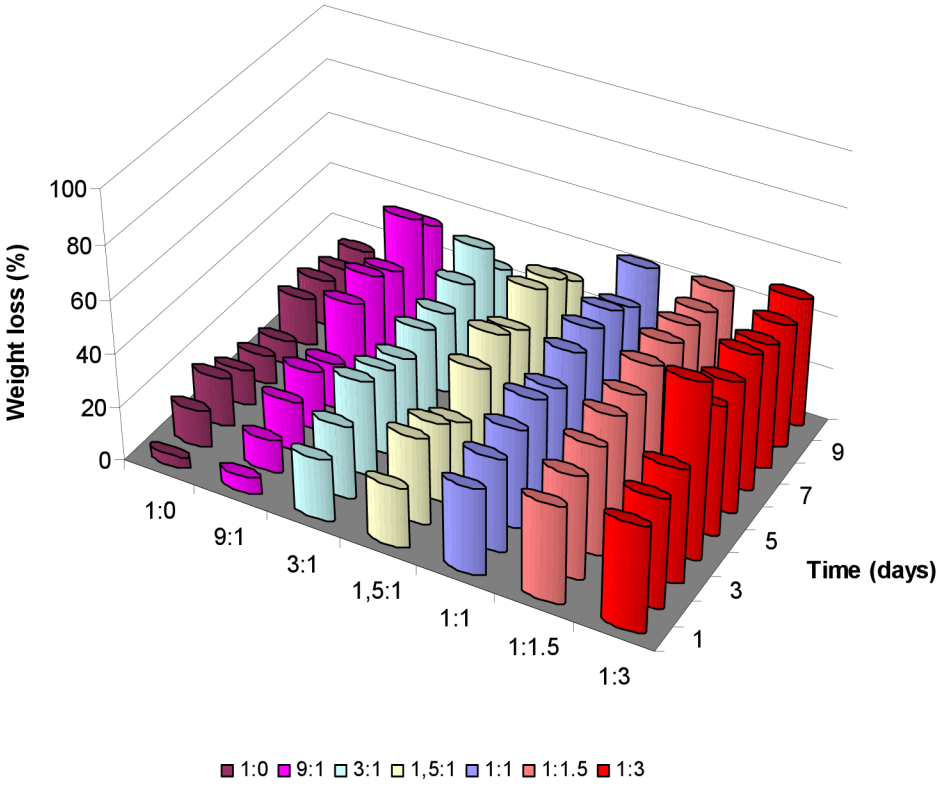

Figure 23 *The dependence of the weight loss on the degradation time for modified and unwashed collagen/cellulose mixture.*

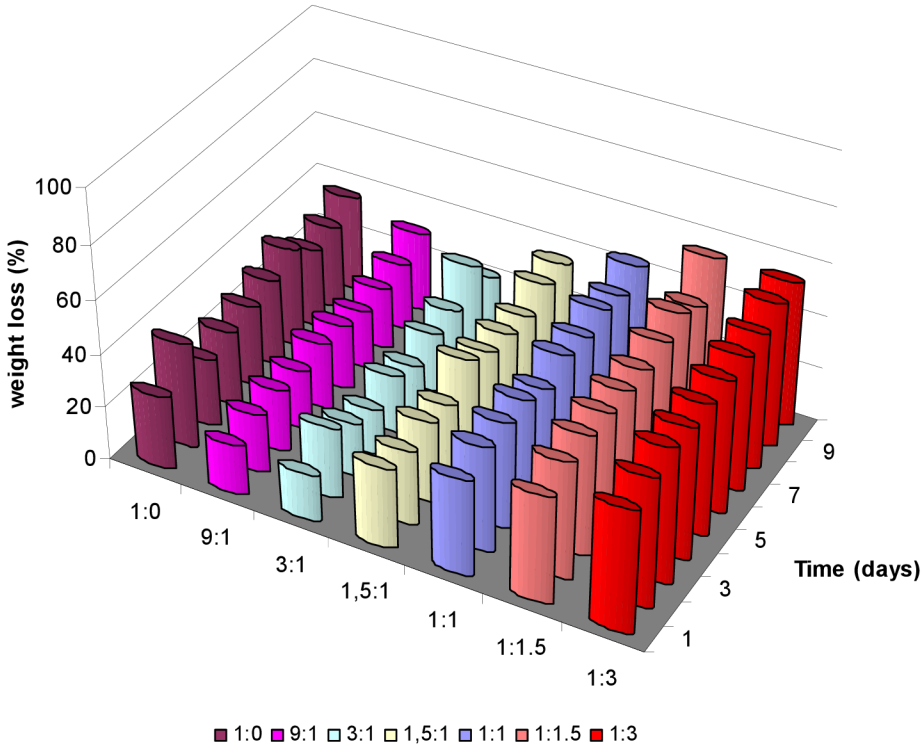

Figure 24 *The dependence of the weight loss on the degradation time for modified and washed collagen/cellulose mixture.*

4.5 Determination of the free amino groups

Colored reactions of the ninhydrin or 2,4,6-trinitrobenzenesulfonic acid (TNBS) with the free amino groups of collagen by UV-VIS spectroscopy were evaluated in order to see the cross-linking or degradation of collagenous samples.

4.5.1 Ninhydrin method

4.5.1.1 Calibration

The results are given in Table 6 and calibration curve is shown in Figure 25. The resulting concentrations of the samples were calculated from the regression equation, where y = 61.758x – 0.0369.

Table 6 Calibration of lysine using ninhydrin method.

Initial concentration (mg/ml)	Absorbance λ = 570 nm	Concentration of amino groups (mg/ml)
0.001	0.060	0.0016
0.002	0.067	0.0017
0.003	0.161	0.0032
0.004	0.170	0.0033
0.006	0.341	0.0061
0.007	0.412	0.0073
0.008	0.405	0.0072
0.009	0.538	0.0093
0.010	0.602	0.0103

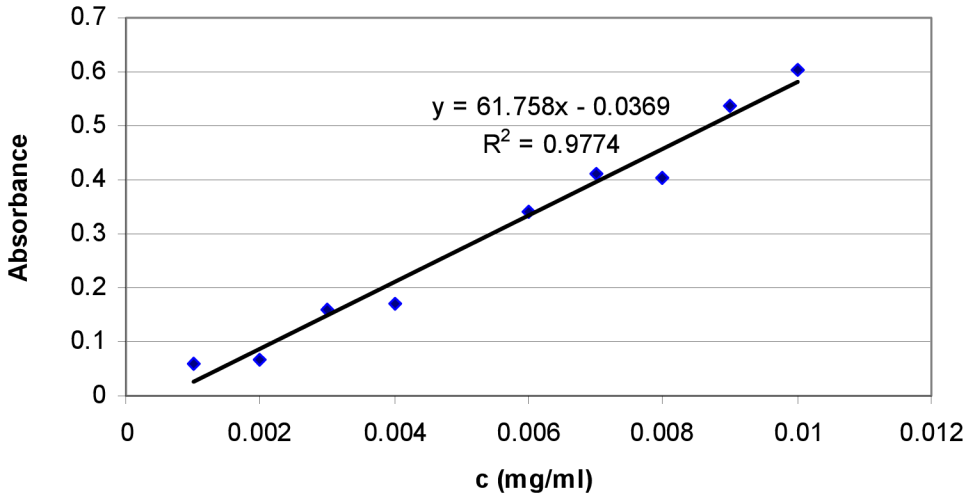

Figure 25 *Calibration curve of lysine determined using ninhydrin method.*

4.5.1.2 Pure collagen/cellulose mixtures

Firstly, the pure collagen/cellulose mixtures were established. Resulting values are given in the Table 7 and graphic progression is in the Figure 26.

By the ninhydrin method it was found that pure collagen had 0.3 % of free amino groups. If the small amount of cellulose was added the percentage of amino groups increased and in the ratio of 9:1 achieves value of 0.9 %. Then the values decreased until the ratio of 5:3 and continue increasing behavior. In the ratio of 1:9 the samples reached the free amino groups amount of 2.2 %. It looks like cellulose reacts with ninhydrin to produce colored product, but from the

Table 7 it is evident that cellulose does not give any positive reaction. As it is described above the cellulose have a stabilizing effect to collagen up to ratio of 1:1, which is confirmed by the decreasing amount of free amino groups. At the ratio higher than 1:1 up to 1:9 the free amino groups increased. This is probably due to fact that the cellulose acts as a solvent of the collagen to make accessible another amino groups of collagen for the colored reactions.

Table 7 Measured and calculated values of the pure collagen/cellulose mixtures determined using ninhydrin method.

Ratio collagen/cellulose	Absorbance	Free amino groups (%)	Standard deviation
1:0	0.002	0.314	0.015
9:1	0.079	0.924	0.295
3:1	0.030	0.534	0.224
5:3	0.012	0.388	0.026
1:1	0.084	0.964	0.300
1:2	0.140	1.408	0.109
1:3	0.125	1.296	0.144
1:9	0.242	2.225	0.282
0:1	-0.005	0	0

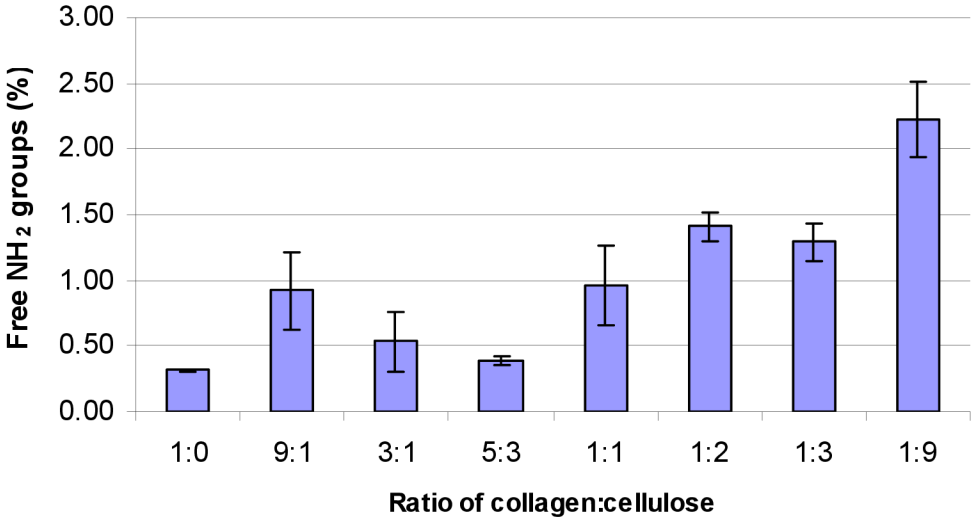

Figure 26 *Pure collagen/cellulose mixtures determined using ninhydrin method.*

4.5.1.3 Collagen/cellulose mixtures activated in situ

In the next step the mixtures of collagen/cellulose were activated in the solution (in situ).The resulting values and graphic progression (Figure 27) are comparable with the values of unmodified collagen/cellulose mixtures. A difference is only in the absorbance intensity, which is higher at the modified samples because the cross-linking EDC agent gives positive reaction with ninhydrin.

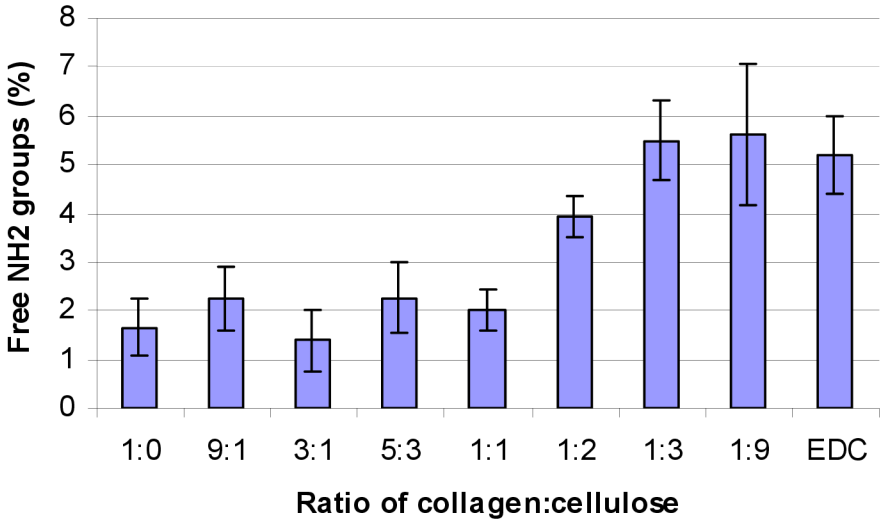

Figure 27 *Activated collagen/cellulose mixtures in situ determined using ninhydrin method.*

4.5.1.4 Collagen/cellulose mixtures activated in the film

Due to very difficult dissolving prepared films (Figure 28) the results (Figure 29) were affected and show for several ratios significant deviations.

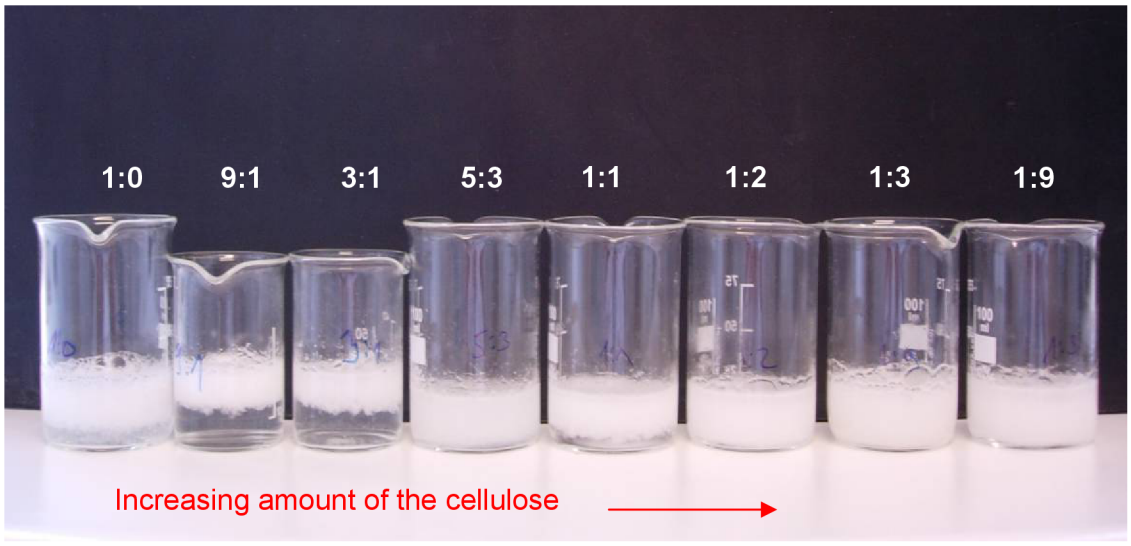

Figure 28 *Dissolved films in the water.*

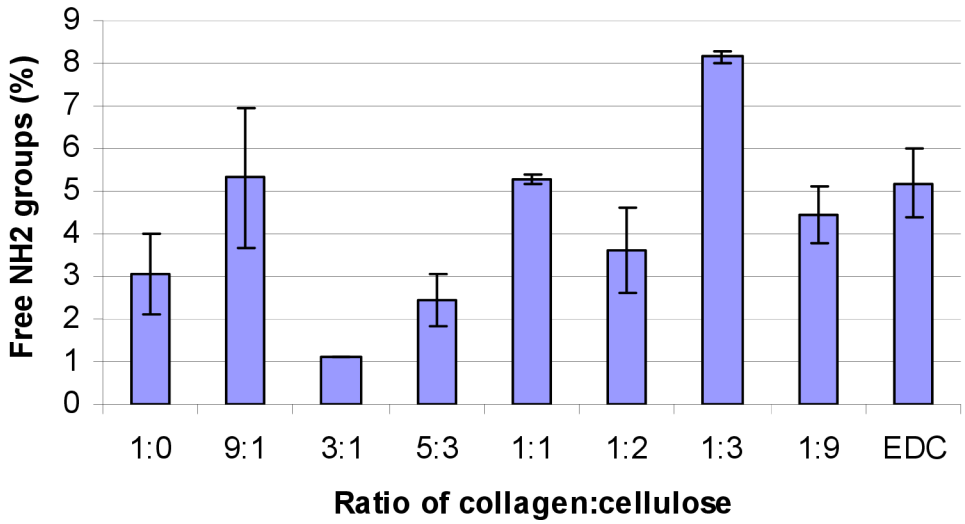

Figure 29 *Activated collagen/cellulose mixtures in the film determined using ninhydrin method.*

4.5.1.5 Collagen/cellulose mixtures with addition of sodium dodecyl sulfate

To prevent precipitation the 10% solution of SDS was added to the polymer mixtures. The resulted mixtures with addition of SDS were not precipitated and were stable. Results of free amino groups' percentage (Figure 30) with addition of SDS are lower than values without addition of SDS. It might be due to fact that the SDS protects the ninhydrin reaction with amino groups. It based on the results that pure collagen with SDS had 0.1 % of free amino groups in comparison with 0.3 % of pure collagen without SDS.

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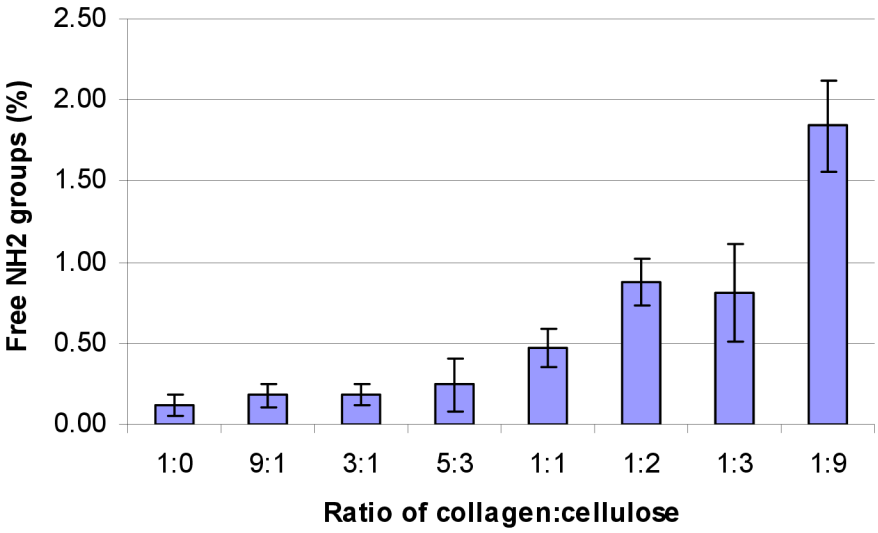

Figure 30 *Collagen/cellulose mixtures with addition of SDS determined using ninhydrin method.*

4.5.1.6 Activated collagen/cellulose mixtures in situ with SDS addition

The mixtures with SDS were additionally activated by EDC/NHS. In the Figure 31 prepared samples are illustrated. During the dissolving prepared films two separate parts were arisen, which influenced the results. All polymer ratios achieve almost same results of about 3 % of free amino groups (Figure 32). The results are much higher due to the positive reaction of EDC with ninhydrine.

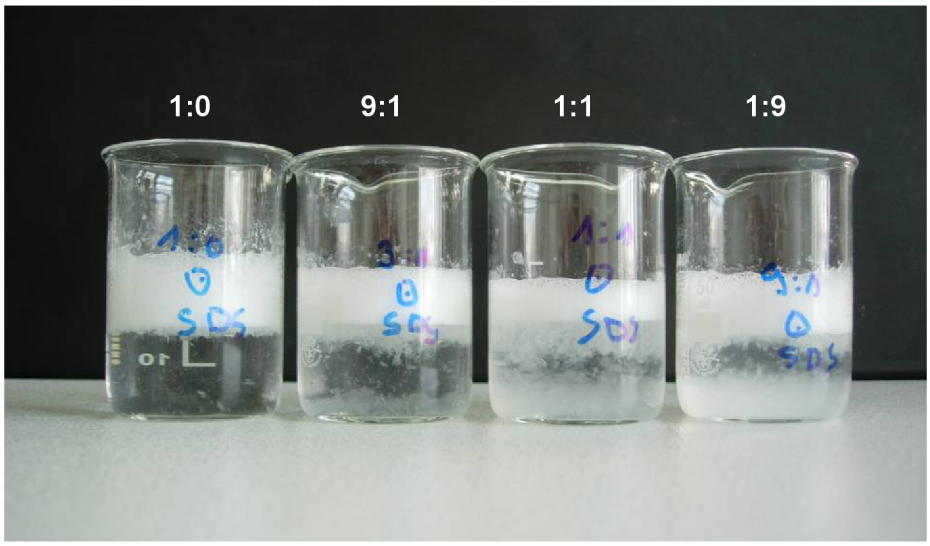

Figure 31 *Prepared solutions from activated films.*

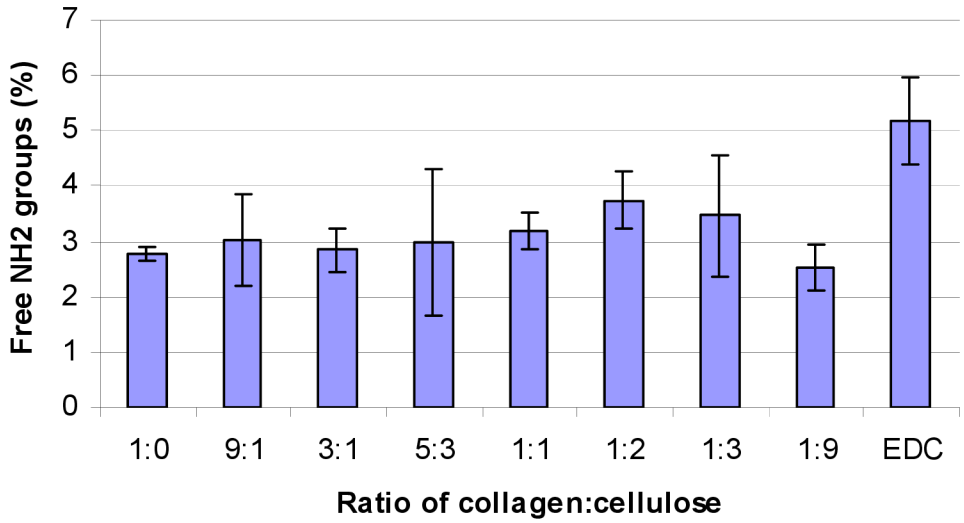

Figure 32 *Activated collagen/cellulose mixtures in situ with addition of SDS determined using ninhydrin method.*

4.5.1.7 Activated collagen/cellulose mixtures in the film with addition of SDS

In the last step films were prepared from the mixtures with SDS, which were activated and subsequently dissolved in water. The pictures of the prepared samples are shown in Figure 33. During the dissolving the films hardly swelled and even were not dissolved in the water. This is the reason of high standard deviation and various results (Figure 34).

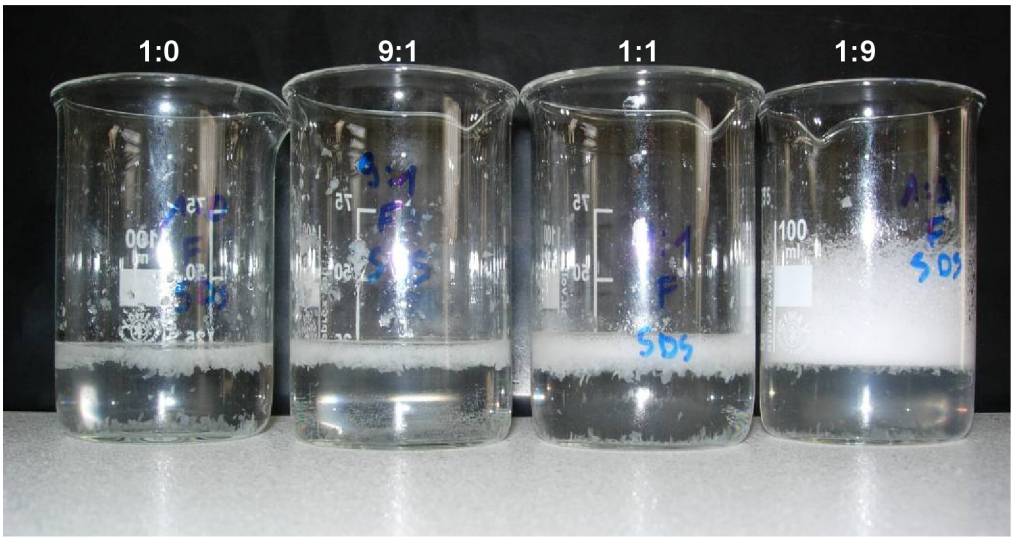

Figure 33 *Prepared solutions from activated films with SDS..*

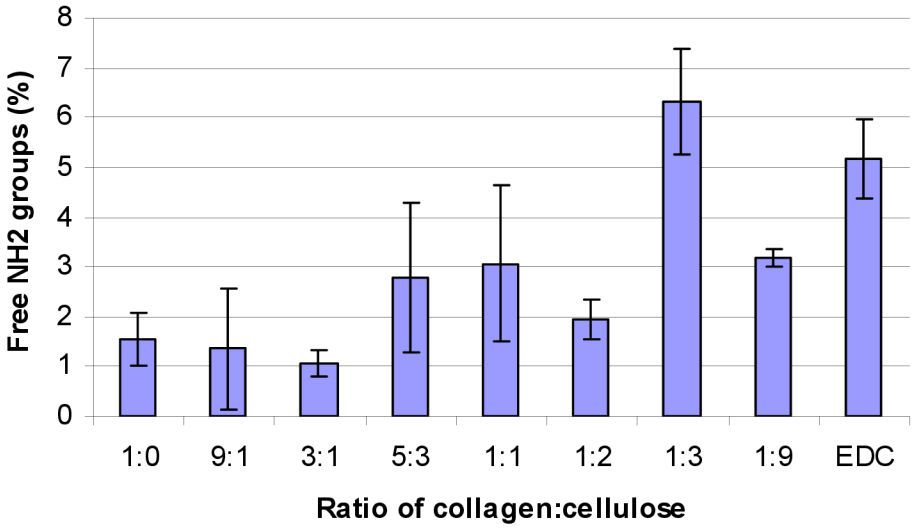

Figure 34 *Activated collagen/cellulose mixtures in the film with addition SDS determined using ninhydrin method.*

4.5.2 2,4,6-trinitrobenzenesulfonic acid reaction (TNBS)

2,4,6-trinitrobenzenesulfonic acid (TNBS) reaction was developed to determine the free amino groups of amino acids and peptides, especially for the determination of ε-amino groups of proteins.

4.5.2.1 Calibration

Amino acid lysine was chosen for the calibration curve. The resulting values are introduced in Table 8 and calibration curve is shown in the Figure 35. The resulting concentrations of the samples were calculated from the regression equation, where y = 41.183x – 0.0118 with factor of reliability 0.9943.

Table 8 Calibration of lysine using TNBS reaction.

Initial concentration (mg/ml)	Absorbance λ = 340 nm	Concentration of amino groups (mg/ml)
0.001	0.043	0.001
0.002	0.090	0.002
0.003	0.147	0.003
0.004	0.185	0.004
0.006	0.214	0.005
0.007	0.270	0.006
0.008	0.324	0.008
0.009	0.382	0.009
0.010	0.428	0.010

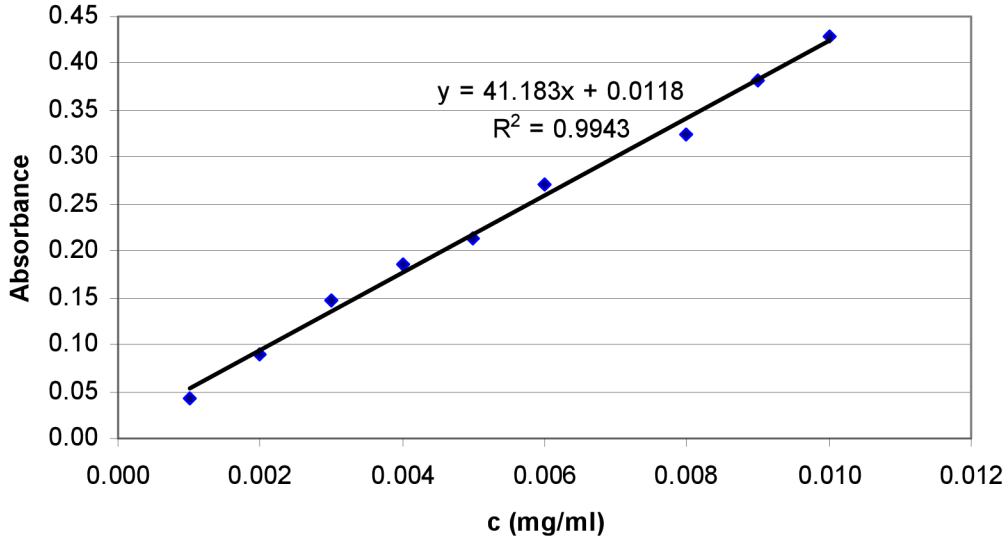

Figure 35 *Calibration curve of TNBS reaction.*

4.5.2.2 Pure collagen/cellulose mixtures

Firstly, the pure collagen/cellulose mixtures were investigated using TNBS analysis. The resulting values (Figure 36) determined by TNBS reactions are comparable with results established by ninhydrin reaction. Both methods have the same behavior of graphic progressions but the intensity of results measured using TNBS method is higher in comparison with values from ninhydrin method. This is due to the positive reaction of cellulose with TNBS, which reach value of 3.1 % of amino group. Pure collagen determined by TNBS method showed 1.5 % of free amino group in comparison with 0.3 % obtained by ninhydrin method resulting in higher sensitivity of TNBS method. In the ratio of 9:1 the percentage of free amino groups reach value of 2.1 % which slowly decreases until the ratio of 5:3. From this ratio the values began increase and in the ratio 1:9 achieve value of 2.6 %.

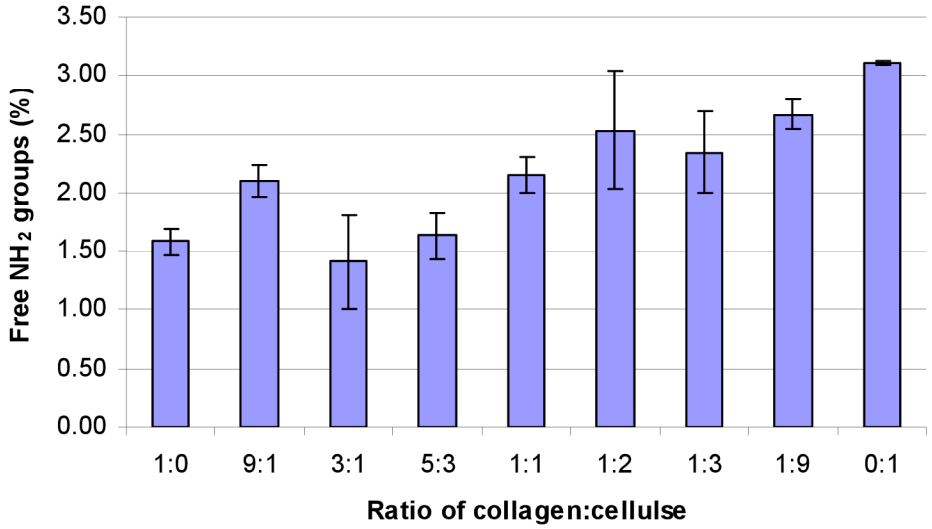

Figure 36 *Pure mixtures of collagen/cellulose determined using TNBS reaction.*

4.5.2.3 Collagen/cellulose mixtures activated in the situ

In the next step the prepared mixtures were activated in the solution. The results are affected by the positive reaction of cellulose and cross-linking agent with TNBS whose value is 5.2 %. The graphic progression (Figure 37) has very similar behavior with the pure mixtures but the intensities of the results are lower. This is due to the cross-linking reaction between collagen end excess of EDC/NHS.

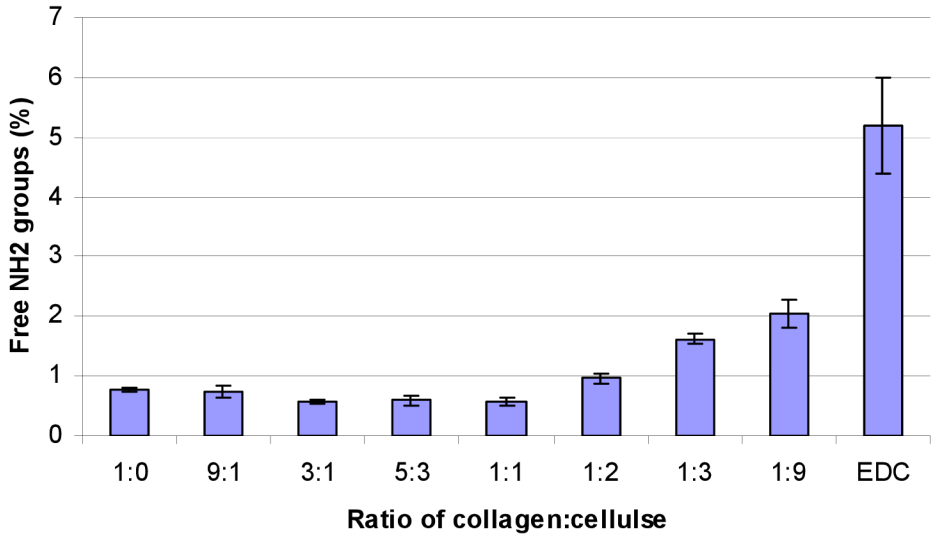

Figure 37 *Activated collagen/cellulose mixtures in situ determined using TNBS reaction.*

4.5.2.4 Collagen/cellulose mixtures cross-linked in the film

As is described above the sample that was cross-linked in the film was very difficult to dissolve in the water, which implies a good stability of mixtures and resistant against hydrolysis. The graphic progression (Figure 38) starts with value of 0.9 % for pure collagen and continues by very small decline of 0.7 % in the ratio of 9:1. The values have same behavior to the ratio of 1:1 and then start increase to value of 2 % in 1:3 ratio. In the ratio of 1:9 the value dropped to the 1.4 % of amino groups.

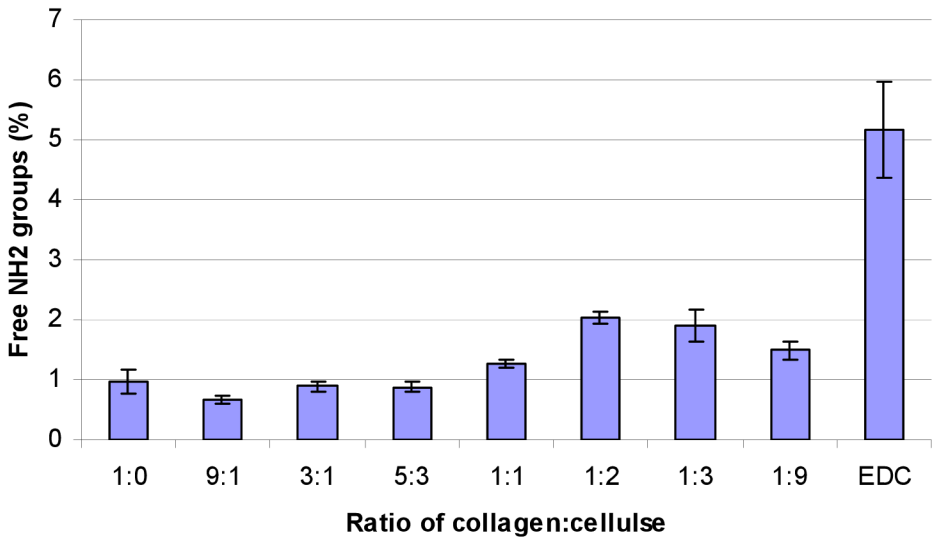

Figure 38 *Activated collagen/cellulose mixtures in the film determined using TNBS.*

4.5.2.5 Collagen/cellulose mixtures with addition of SDS

Due to precipitation of mixtures the SDS was added (Figure 39). The results are influenced by positive reaction of cellulose with TNBS. 3.1 % is the value of amino groups for cellulose. Other samples reach almost same value if the deviation is taking into account. From this it is evident that the SDS protect the amino groups from the reaction with TNBS and mixtures reach the same values as the pure cellulose.

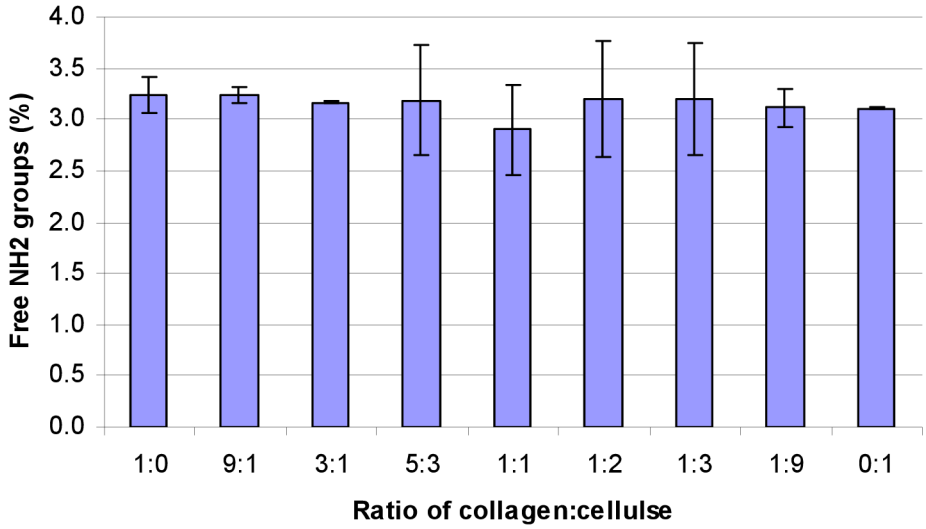

Figure 39 *The collagen/cellulose mixtures with addition of SDS determined using TNBS.*

4.5.2.6 Activated collagen/cellulose mixtures in the situ with addition of SDS

The data for the samples activated in the solution are given in the Figure 40. Results are comparable with the results of the samples activated without addition of SDS. The results are very little affected by the positive reaction of EDC/NHS with TNBS because the abundance was removed by washing the samples. If the standard deviation is speculated the ratio of 1:2, 1:3 and 1:9 have almost same value of 1.3 % of amino groups. The ratios from 1:0 to 1:1 have percentage of amino groups between 0.7 - 1 %.

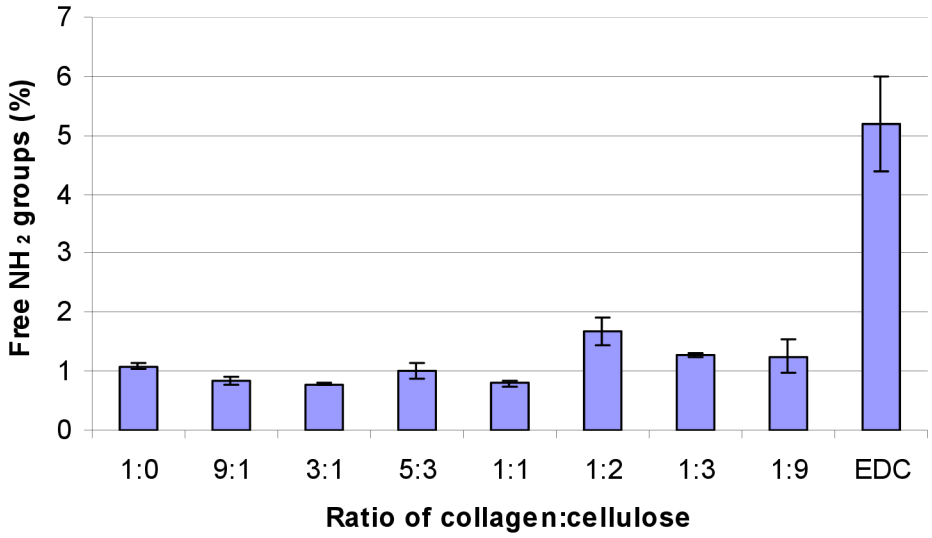

Figure 40 *Activated collagen/cellulose mixtures in the solution with addition of SDS determined using TNBS.*

4.5.2.7 Activated collagen/cellulose mixtures in the film with addition of SDS

Mixtures cross-linked in the film have the comparable results (Figure 41) with the results of sample cross-linked in the solution. The ratios of 5:3 and 1:3 have the highest values equal to 1.2 % of amino groups in comparison with other. The ratio of 9:1, 3:1 and 1:2 have the percentage of amino groups of about 0.5 % and ratio of 1:1 and 1:9 have 1 % of amino groups.

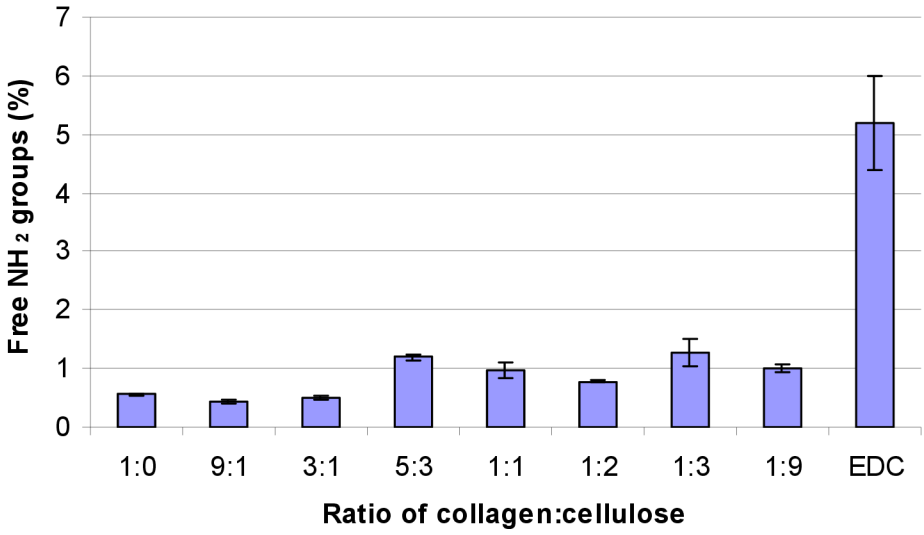

Figure 41 *Activated collagen/cellulose mixtures in the film with addition of SDS determined using TNBS.*

4.6 Characterization by Fourier-transformed infrared spectroscopy

4.6.1 Pure samples

The expected types of bond linkages presented in the samples were confirmed using infrared analysis. Pure collagen gel (1:0) and pure oxidized cellulose solution (0:1) were used for film preparation and evaluation using FT-IR spectroscopy see in Figure 42 and Figure 43, respectively.

Collagen displays bands at 3326, 1658, 1548 and 1236 cm^{-1}, which are characteristic for the amide A, I, II and III bands of collagen, respectively. 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 complex, consisting of components from C–N stretching and N–H in plane bending from amide linkages.

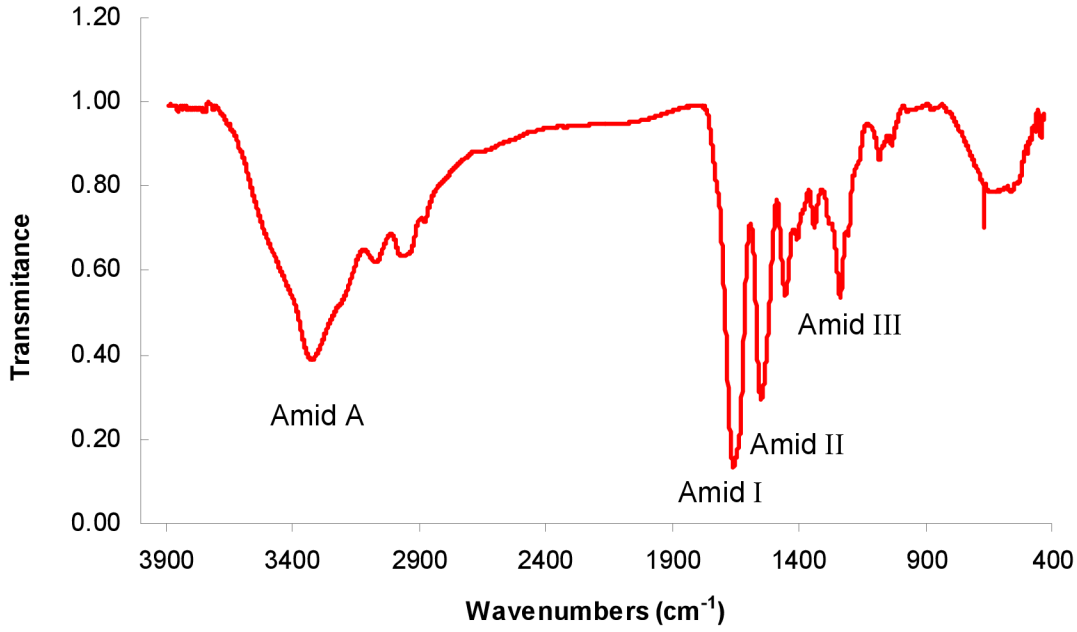

Figure 42 *IR spectrum of pure collagen.*

IR spectrum of oxidized cellulose showed a broad absorption band at 3379 cm^{-1}, that confirms the stretching frequency of the –OH group. The band at 2902 cm^{-1} confirms C–H stretching vibration. The presence of a strong absorption band at 1612 cm^{-1} confirms the presence of COO– group. The bands around 1412 and 1293 cm^{-1} are assigned to –CH2 scissoring and –OH bending vibration, respectively. The band at 1060 cm^{-1} is due to CH_2–O–CH_2 stretching

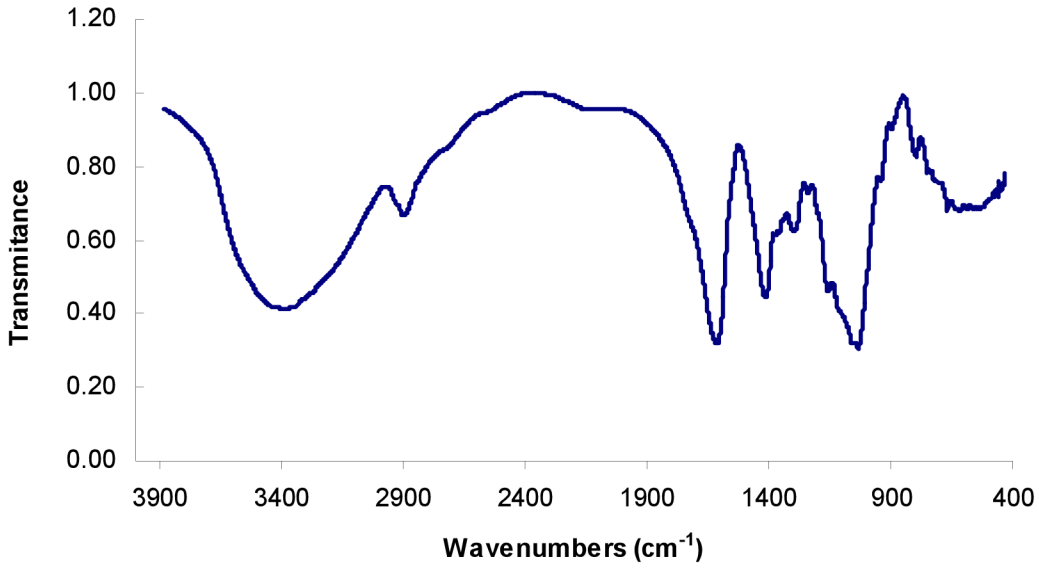

Figure 43 *IR spectrum of oxidized cellulose.*

4.6.2 Spectra of collagen/cellulose mixtures

The blends of collagen/cellulose (pure in Figure 44, activated in situ in Figure 45, activated in the film in Figure 46) were investigated by FT-IR. The frequencies at which major peak occurred for individual mixtures of collagen/cellulose are summarized in Table 9.

From the table it is obvious that the major peak of mixtures have the same resulting position with small deviations. Only for the unmodified collagen with increasing content of cellulose the position of amid I decrease to ratio of 1:1. From this ratio the position starts increase to value 1659 cm^{-1}, which is same as for pure collagen. Because the amide I represents the secondary structure of the collagen, in the next part the amide I peak components were investigated using peak deconvulation.

Table 9 IR spectra peak position of the individual ratio collagen/ cellulose

Types	Amid A [cm^{-1}]	Amid I [cm^{-1}]	Amid II [cm^{-1}]	Amid III [cm^{-1}]
Unmodified				
1:0	3324	1659	1548	1236
9:1	3329	1659	1549	1236
3:1	3330	1657	1545	1233
5:3	3333	1633	1547	1235
1:1	3335	1617	1550	1236
1:2	3335	1615	1551	1236
1:3	3341	1625	1549	1237
1:9	3330	1659	1548	1236
Activated in solution				
1:0	3322	1657	1542	1231
9:1	3323	1655	1543	1232
3:1	3318	1650	1537	1231
5:3	3330	1656	1542	1232
1:1	3331	1656	1545	1233
1:2	3335	1657	1547	1235
1:3	3335	1656	1547	1235
1:9	3342	1652	1551	1235
Activated in films				
1:0	3326	1655	1550	1236
9:1	3325	1659	1550	1236
3:1	3326	1657	1548	1235
5:3	3331	1656	1549	1235
1:1	3334	1656	1551	1236
1:2	3337	1656	1552	1236
1:3	3337	1659	1551	1236
1:9	3338	1632	1554	1236

With increasing content of the cellulose in polymer blends the intensity of amid A increases because increase the content of –OH groups in the mixtures. If the mixtures are activated by EDC/NHS and the values of pure collagen with other ratios are compared, the intensity of amid A confirming $–NH_2$ decrease, because the amino groups react with activated carboxyl group of collagen.

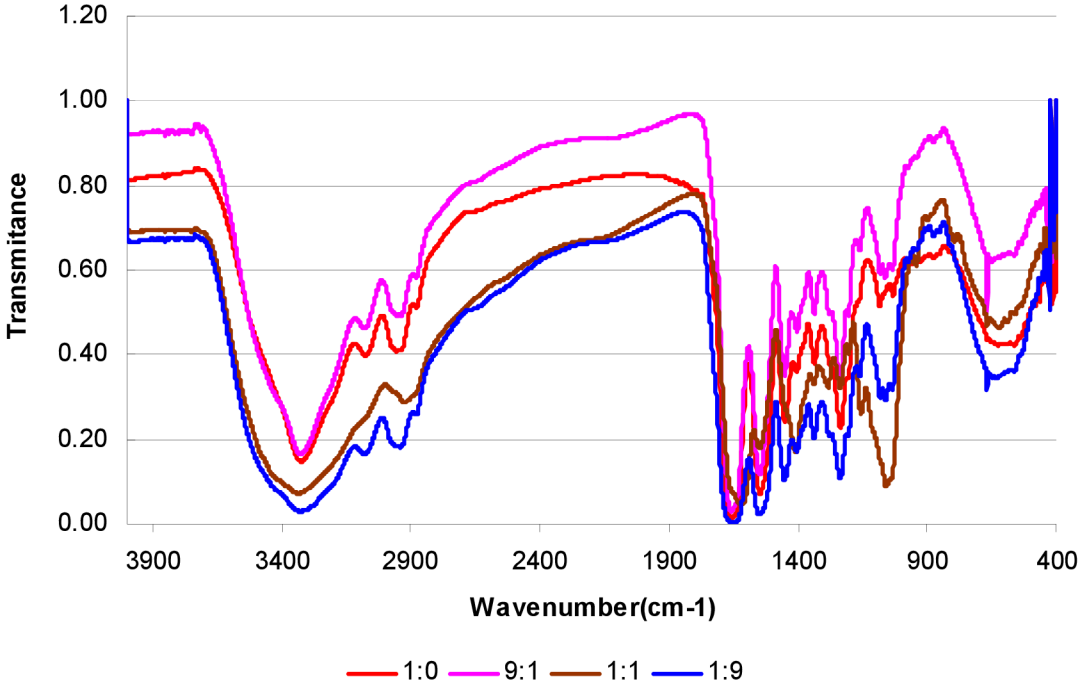

Figure 44 *IR spectra of unmodified collagen/cellulose blends.*

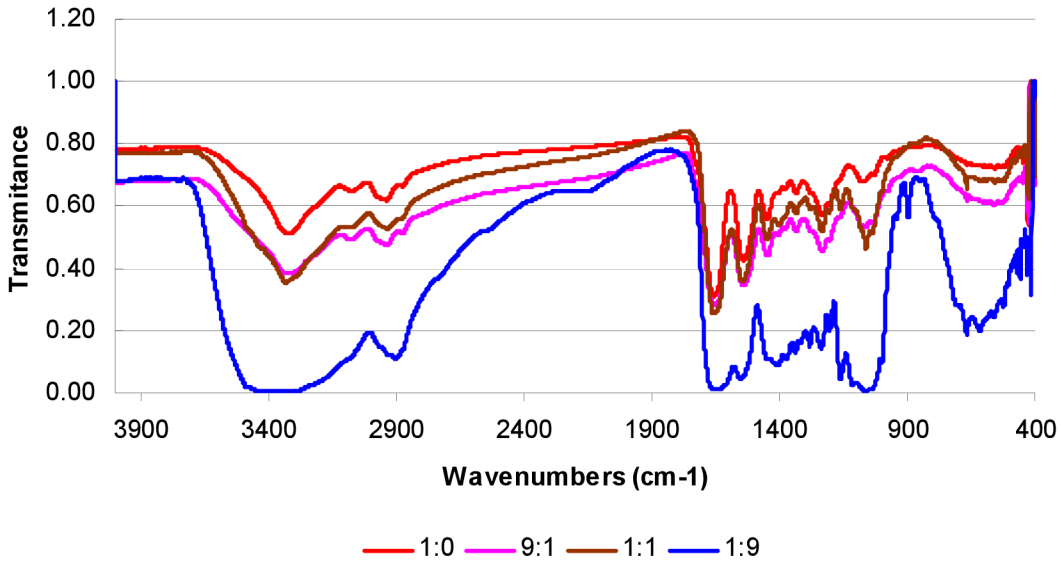

Figure 45 *IR spectra of collagen/cellulose blends activated in situ.*

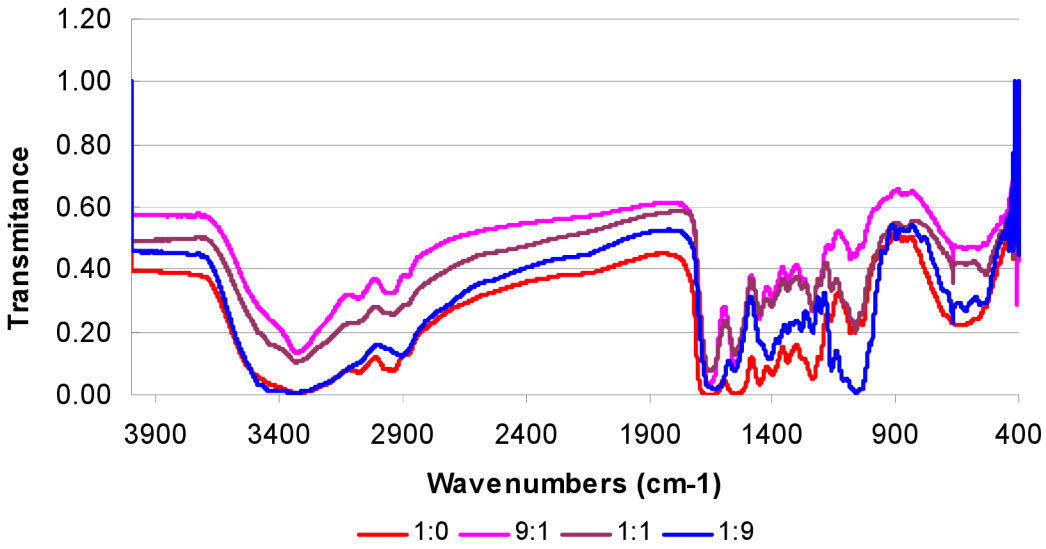

Figure 46 *IR spectra of collagen/cellulose blends activated in the film.*

4.6.3 Amid I band components of a collagen/cellulose mixtures

This part was focused on the study of changes in collagen secondary structure in dependence on addition of cellulose into the collagen mixture. The amide I band, between 1600 and 1700 cm^{-1}, is the most useful for infrared spectroscopic analysis of the protein. Deconvolution of the amide I band of unmodified and activated mixtures showed that the band consists of four components (Figure 47). Denaturation of collagen was established from the measured values after addition of oxidized cellulose. Denaturation of collagen is characterized by increase in the intensity of amide I component found around 1630 cm^{-1} and reduction in the intensity of amide I component found around 1660 cm^{-1} [13]. Therefore with the increasing value of intensity ratios of peaks 1630/1660 the denaturation supposed to increase. The dependence of intensity 1630/1660 ratio on the ratio of collagen/cellulose is shown in the Figure 48

The resulting values are shown in appendix A 1 and A 2and graphs of deconvulation on appendix from A 3 to A 9 of pure mixtures. The results of mixtures activated in the solution are introduced in appendix A 10 and A 11 and graph from A 12 to A 19. The results of mixtures activated in the film are introduced in appendix A 20 and A 21 and graph from A 22 to A 29.

The behaviors from this measurement are very similar and comparable with UV spectroscopic measurement. Pure collagen reaches the 1630/1660 value of 0.6 and after addition of cellulose this value increase to 1.2 in the ratio of 9:1. If the next amount of cellulose was added the collagen became stable which is confirmed by the decrease in the intensity ratio of 1630/1660. With next addition of cellulose the intensity ratio increase and in the ratio of 1:9 reach the value of 1.6.

The collagen/cellulose mixtures activated in the film have similar behavior as the pure mixture only in the ratio of 1:9 the value is equal to 0.4. The collagen/cellulose mixtures activated in situ have decreasing behavior.

Based on the results it is evident that the stabilizing effect of cellulose to collagen is extinguished by the cross-linking agent of EDC/NHS because the cellulose have only 0.6 % of carboxyl groups able to react with collagen.

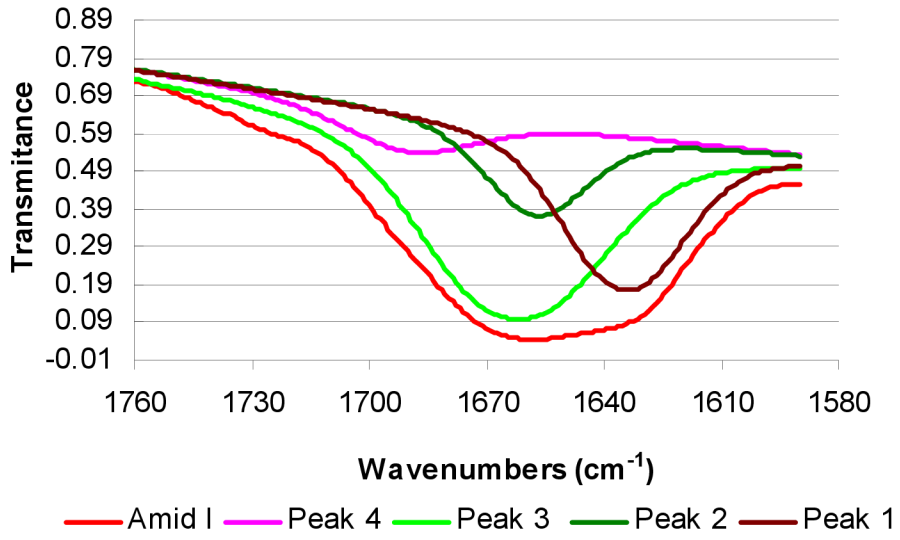

Figure 47 Amid I band of pure collagen/cellulose mixtures in ratio of 1:0 with fitted components.

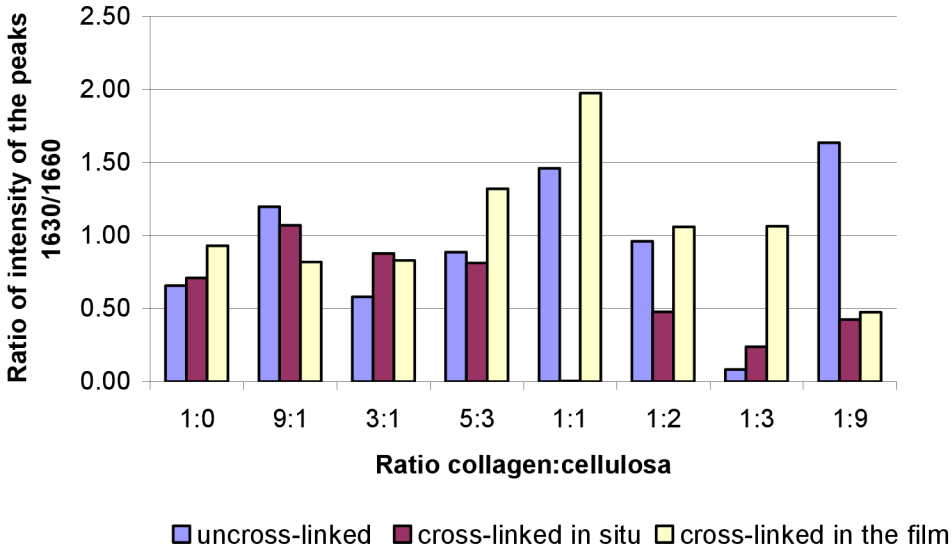

Figure 48 *The dependence of intensity peaks 1630/1660 ratio on collagen/cellulose ratio.*

4.7 Morphology of collagen/cellulose scaffolds

For the investigation of porosity and morphological changes two types of collagen/cellulose mixtures were prepared. First type was frozen slowly at –30 °C in the freezer and second type was frozen quickly using liquid nitrogen at -196 °C. The freezing

process initially produced the nuclei of ice that increased to final size of ice crystal and produce a pore in the matrix of collagen or cellulose. The frozen samples were subsequently freeze-dried under vacuum. The porosity and pore size were investigated using ImageJ software.

Graph of porosity and pore size are shown in the Figure 49 and Figure 49 respectively. The resulting values are introduced in appendix A 30 for mixtures frozen using nitrogen and in appendix A 31 for mixtures frozen in the freezer.

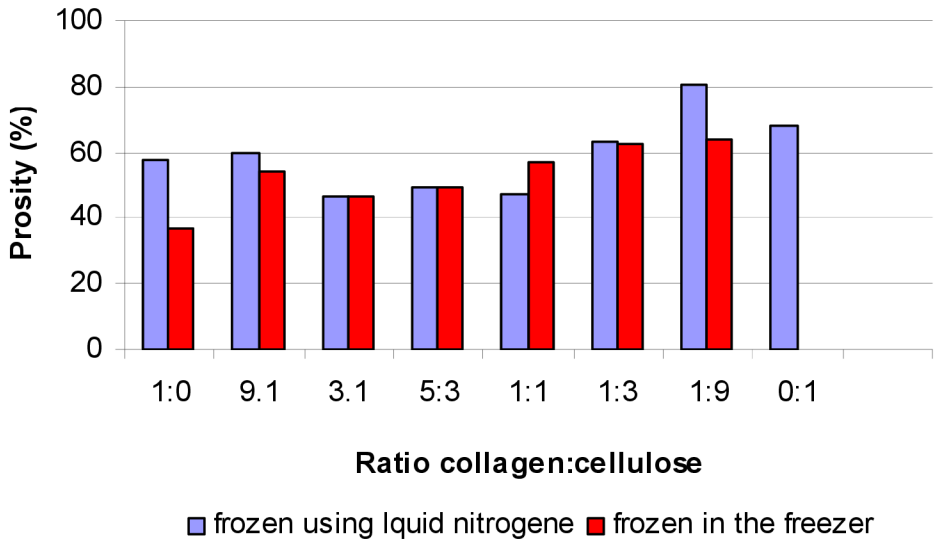

Figure 49 *Porosity of the collagen/cellulose mixtures frozen using liquid nitrogen or in the freezer.*

The porosity of mixtures frozen in the freezer and using liquid nitrogen is comparable up to collagen/cellulose ratio of 1:3 and reaches values between 50 and 60 %. Only the ratio of 1:9 and pure cellulose show higher porosity. If the 1:9 ratio was frozen in the freezer the porosity reach value of 64 % and pore size value of 57± 25 μm. If the ratio of 1:9 was frozen using liquid nitrogen the porosity was equal approx. to 80 %. The pure cellulose scaffolds reaches porosity of 69 %. The porosity of the 1:9 ratio and pure cellulose is very regular and has higher pore size than other samples (Figure 51 and Figure 52)

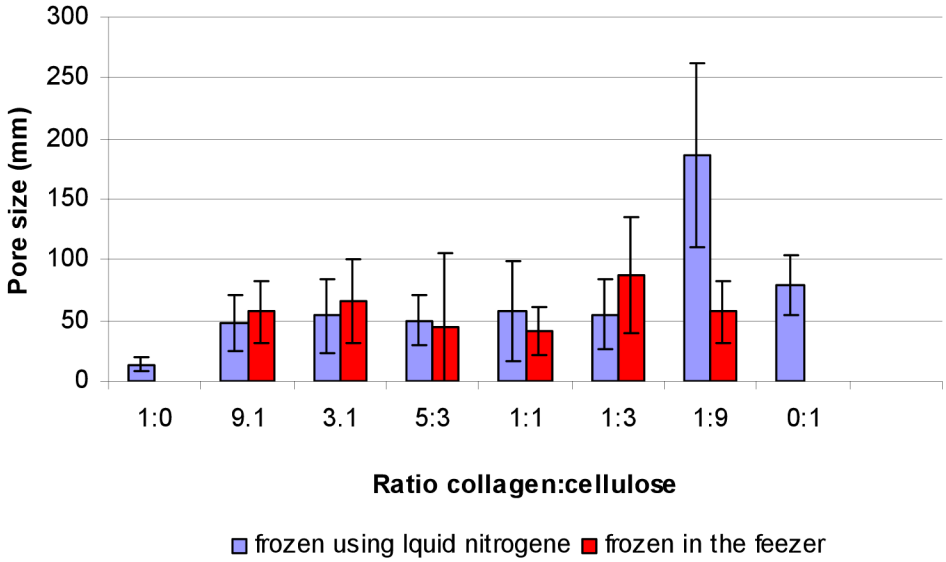

Figure 50 *Pore size of the collagen/cellulose mixtures frozen using liquid nitrogen or in the freezer.*

Average pore size of neat collagen (Figure 53 and Figure 54) was very small (14 ± 5 μm) in comparison with pure oxidized cellulose (79 ± 24 μm). That is why the cellulose addition increased the pore size approximately up to 55 μm except for the 1:9 ratio having pore size about 186 ± 76 μm. Based on the results it can be deduced that the freezing temperature did not have effect on the porosity and pore size of the mixtures having ratio of 1:9 at which both parameters significantly increased.

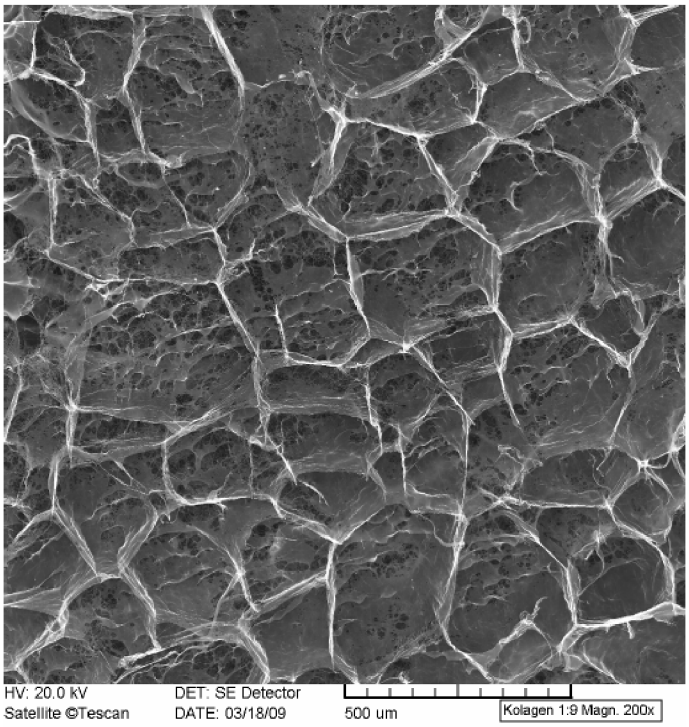

Figure 51 *Morphology of the collagen/cellulose ratio of 1:9 observed by SEM and frozen using liquid nitrogen.*

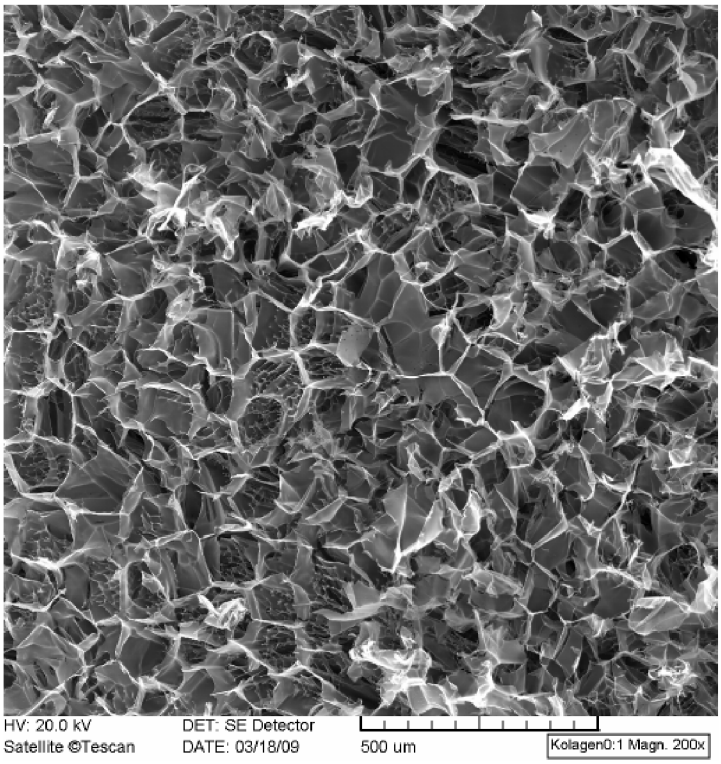

Figure 52 *Morphology of the collagen/cellulose ratio of 0:1 (neat cellulose) observed by SEM and frozen using liquid nitrogen.*

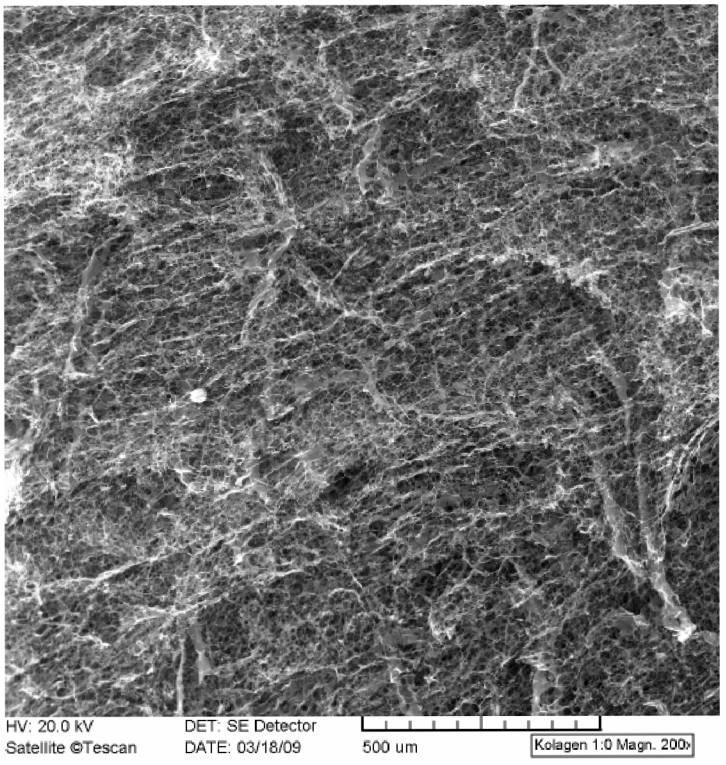

Figure 53 *Morphology of the collagen/cellulose ratio of 1:0 (pure collagen) observed by SEM and frozen using liquid nitrogen.*

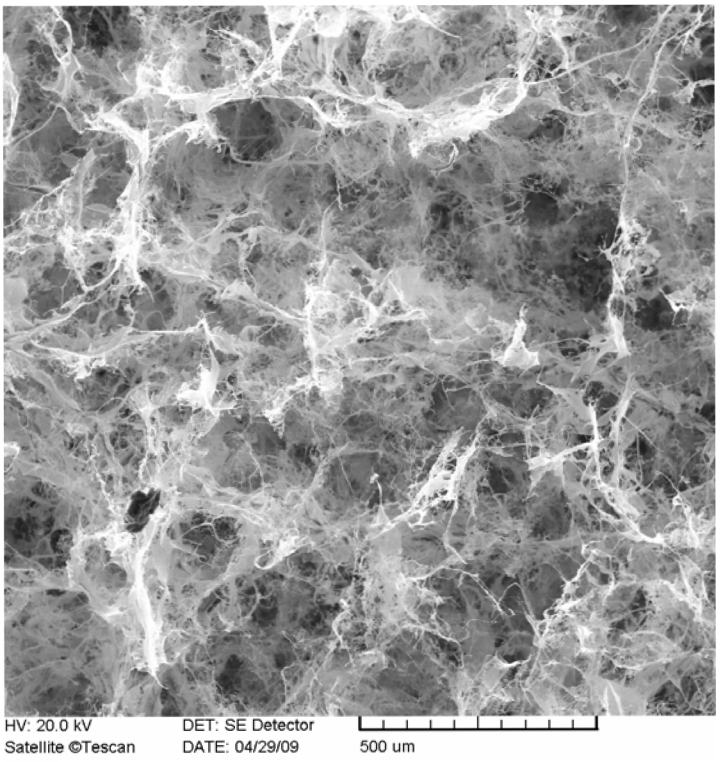

Figure 54 *Morphology of the ratio collagen/cellulose ratio of 1:1 observed by SEM and frozen in the freezer.*

5 CONCLUSION

Collagen as a most widespread protein in animal field has a wide use in biomedical application. The aim of this work was to extend the use of collagen and investigate its behavior in a mixture with oxidized cellulose, which have a carboxyl groups able to react with free amino groups of collagen in order to form amid bonds.

Firstly, the series of collagen/cellulose ratios with increasing content of oxidized cellulose and with the same amount of collagen were prepared. Even the small addition of cellulose caused the fast precipitation of the mixture. With the increasing amount of cellulose intensity of the precipitation decrease and in ratio of 1:1 collagen/cellulose the mixtures stopped precipitation. It can be concluded that the addition of small amount of cellulose leads to the emergence of weak physical interactions between collagen and cellulose, which have stabilizing effect to collagen. After the addition of abundance of cellulose to the collagen mixture the cellulose probably does not react with collagen but acts as a solvent for the collagen, which was proved as well by the degradation stability measurement in water at 37 °C. Based on the results, the pure collagen slowly degraded with increasing time of degradation and after 10 days reached 66 % of weight loss. If the cellulose was added the collagen was stabilized and the weight loss reached 28 % at the ratio of 9:1. This trend continued to ratio of 1.5:1. It is evident that after small addition of cellulose to collagen increased the mixture stability. These collagen/cellulose ratios can be used as wound healing coverings material due to the demonstrated higher stability than that of pure collagen. When higher amount of cellulose is mixed with collagen, the weight loss increased and in the ratio of 1:3 reached almost same value as the pure collagen (62 %). On the other hand, these materials can be used as haemostatic covers which do not need long stability. The behaviors were very similar when the EDC/NHS was used.

For the investigation of the chemical reaction between the carboxyl groups of oxidized cellulose and free amino groups of collagen two spectroscopic methods with ninhydrin and 2,4,6-trinitrobenzenesulfonic acid (TNBS) reagents were used. When comparing both methods the more sensitive results give the method using the TNBS. This is evident from the results of pure collagen when the value of free amino groups determined using ninhydrin was about 0.3 % and by TNBS about 1.5 %. Disadvantages were found in positive reactions of both reagents with cross-linking agent and positive reaction of TNBS with cellulose, which very significantly influenced the results. Both methods gave the same trends. When the cellulose was added the free amino groups decreased up to ratio of 1:1 followed by increasing in the amount of the free amino groups with higher cellulose contain. This behavior confirms the statement as is described above. If the EDC/NHS was used as the activator of the carboxyl groups the stabilizing effect of cellulose to collagen is extinguished because the cellulose have only 0.6 % of carboxyl groups able to react with collagen. Thus the activation was not proved.

From the FT-IR spectra of prepared films the amide I band between 1580 -1760 cm^{-1} was investigated in order to characterize the changes in secondary structure of collagen. After the

deconvulation of amide I peak the four new peaks were obtained. Based on the intensities ratios of 1630 cm^{-1} to 1660 cm^{-1} deconvulated peaks, comparable behaviors with UV-VIS spectroscopic methods were seen.

Morphological changes based on the porosity and pore size using SEM were studied in samples frozen at – 196 °C and at -30 °C. The porosity of scaffolds were not affected by freezing temperature, the more significant effect had the polymer ratios, actually the amount of cellulose. The porosity between 46 – 60 % was constant up to the ratio of 1:1 but significantly increased with cellulose addition (up to 81 % at ratio of 1:9). It was found that average pore size of pure collagen was very small (14 ± 5 μm) in comparison with pure oxidized cellulose (79 ± 24 μm) resulting in increasing the average pore size of samples when cellulose is added to collagen (approximately up to 55 μm). Only the 1:9 ratio (the highest amount of cellulose in collagen) having large pore size of about 186 μm and very regular structure resembling honeycomb seen at pure cellulose as well. These samples might be used even for seeding cells in tissue engineering.

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7 LIST OF APPENDIXES

7.1 Infrared spectroscopy

7.2 Morphology of collagen/cellulose scaffolds

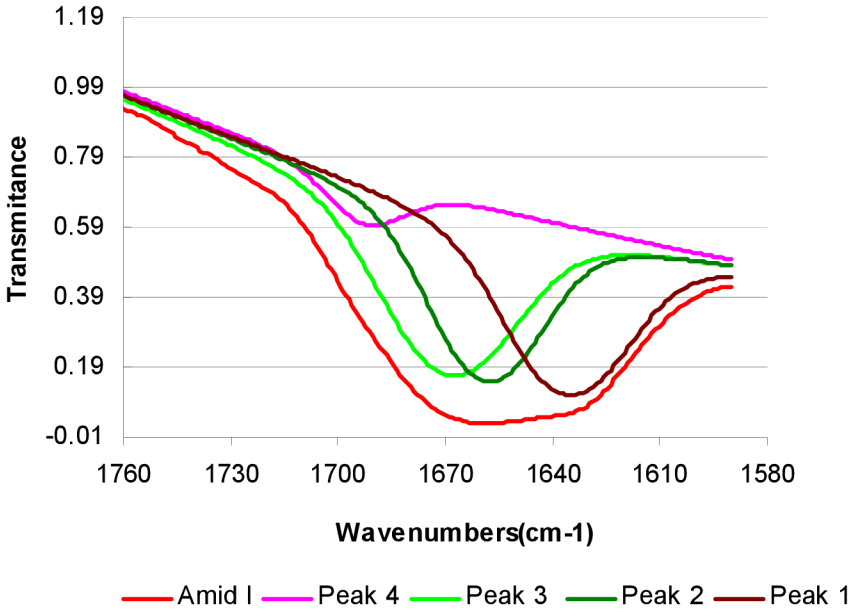
APPENDIX

A 1 Location and percent area contribution of amid I components for the pure collagen/cellulose mixtures (peaks 1 and 2).

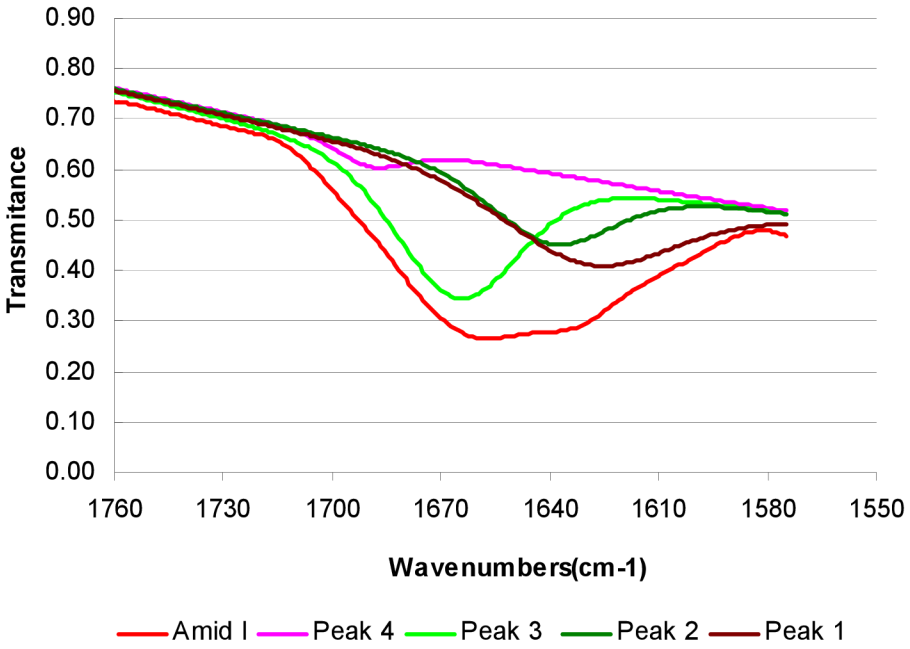
Ratio	Peak 1		Peak 2	
	Peak location [cm^{-1}]	Area	Peak location [cm^{-1}]	Area
1:0	1634.43	20.97	1657.09	8.48
9:1	1635.30	31.42	1657.92	25.61
3:1	1628.10	11.13	1638.95	6.15
5:3	1614.52	19.13	1641.45	12.23
1:1	1611.75	51.13	1638.93	51.13
1:2	1605.04	21.80	1628.81	50.19
1:3	1600.30	2.25	1630.54	12.08
1:9	1633.25	49.66	1658.82	62.61

A 2 Location and percent area contribution of amid I components of the pure collagen/cellulose mixtures (peaks 3 and 4).

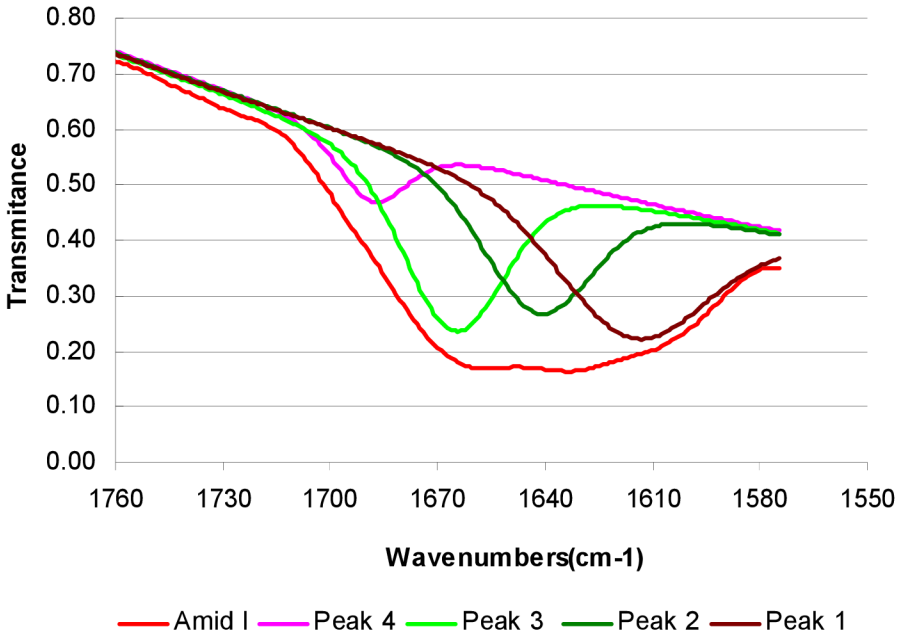
Ratio	Peak 3		Peak 4	
	Peak location [cm^{-1}]	Area	Peak location [cm^{-1}]	Area
1:0	1662.21	43.50	1689.17	4.77
9:1	1668.06	29.35	1692.03	3.43
3:1	1664.85	12.60	1689.02	1.19
5:3	1664.98	14.37	1688.03	3.11
1:1	1665.21	23.43	1688.37	4.46
1:2	1663.96	29.91	1689.55	1.62
1:3	1661.60	27.05	1695.29	0.00
1:9	1676.93	34.72	1694.77	3.76



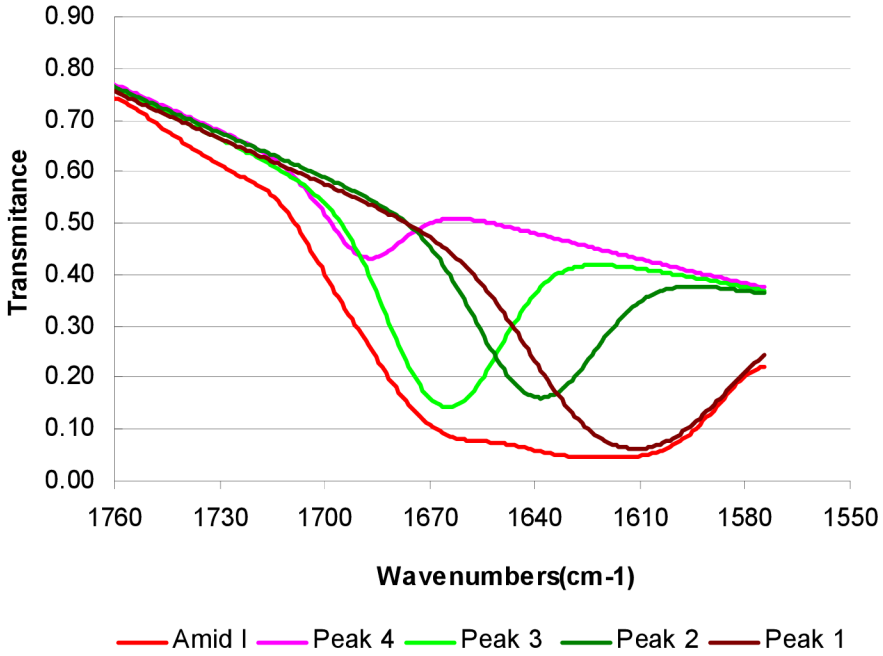
A 3 *Amid I band of pure collagen/cellulose mixtures in ratio of 9:1 with fitted components.*



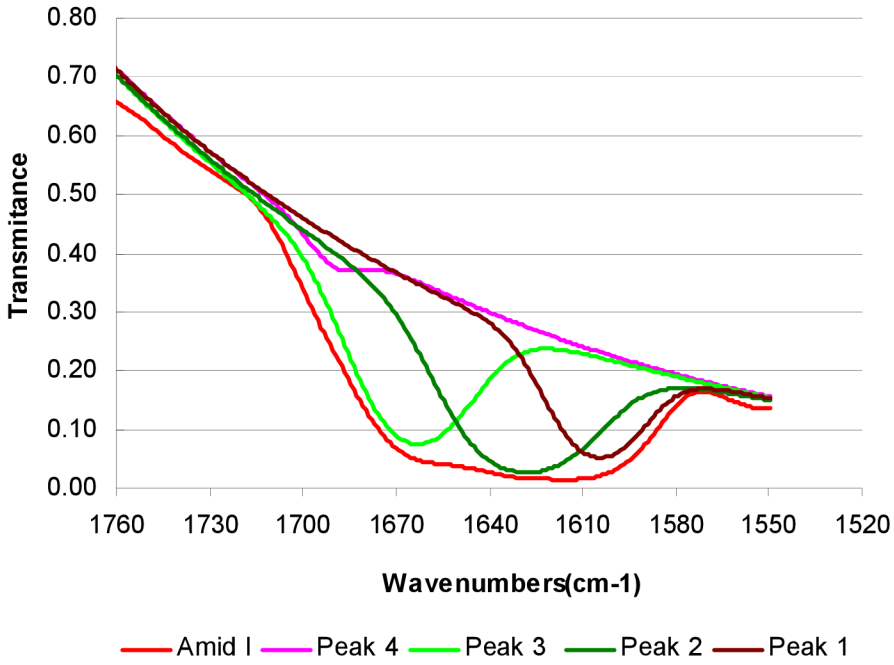
A 4 *Amid I band of pure collagen/cellulose mixtures in ratio of 3:1 with fitted components.*



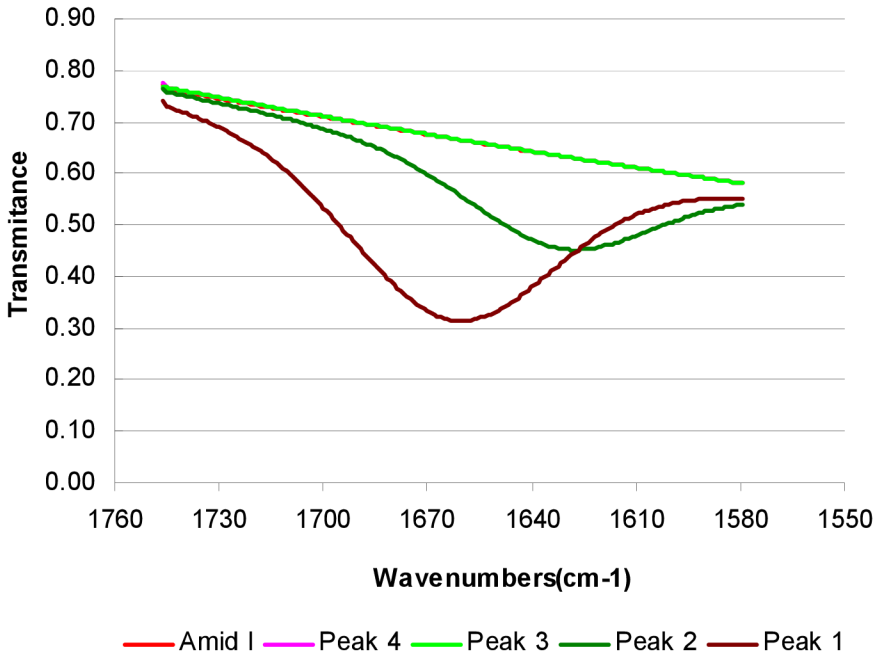
A 5 *Amid I band of pure collagen/cellulose mixtures in ratio of 5:3 with fitted components.*



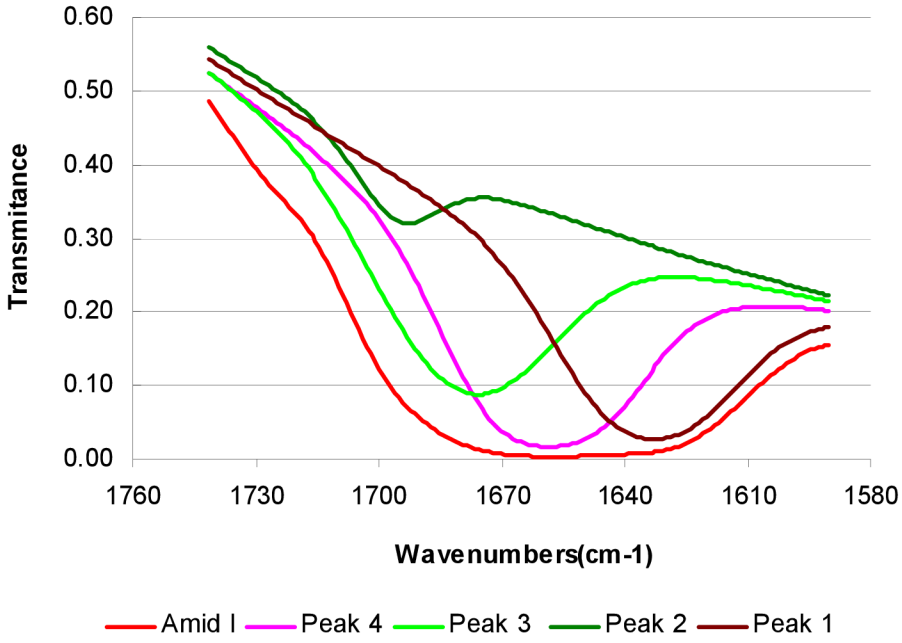
A 6 *Amid I band of pure collagen/cellulose mixtures in ratio of 1:1 with fitted components.*



A 7 *Amid I band of pure collagen/cellulose mixtures in ratio of 1:2 with fitted components.*



A 8 *Amid I band of pure collagen/cellulose mixtures in ratio of 1:3 with fitted components.*



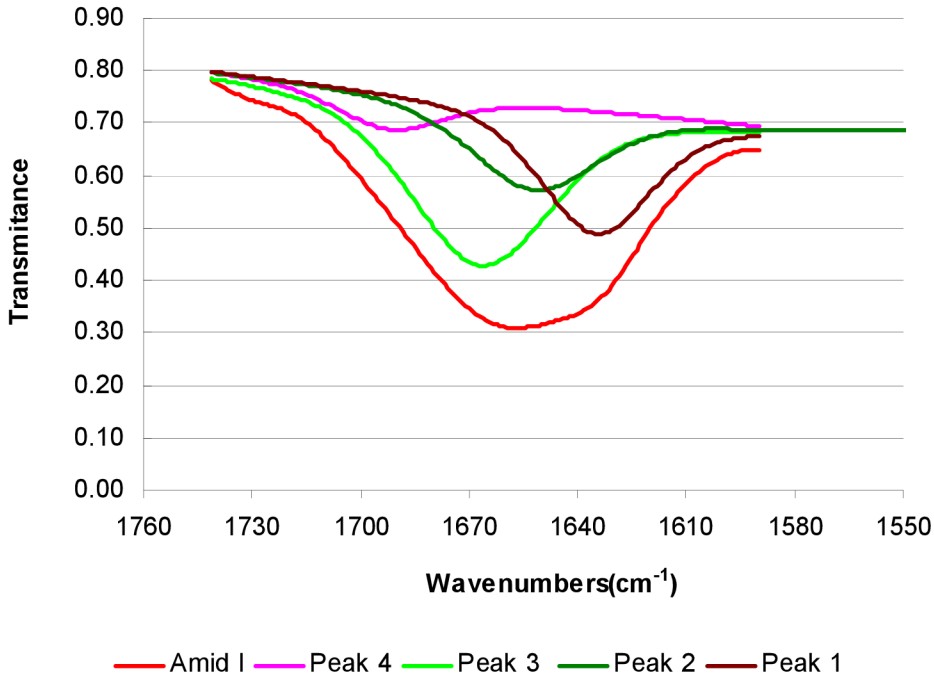
A 9 *Amid I band of pure collagen/cellulose mixtures in ratio of 1:9 with fitted components.*

A 10 Location and percent area contribution of amid I components for the *activated collagen/cellulose mixtures* in situ (peaks 1 and 2).

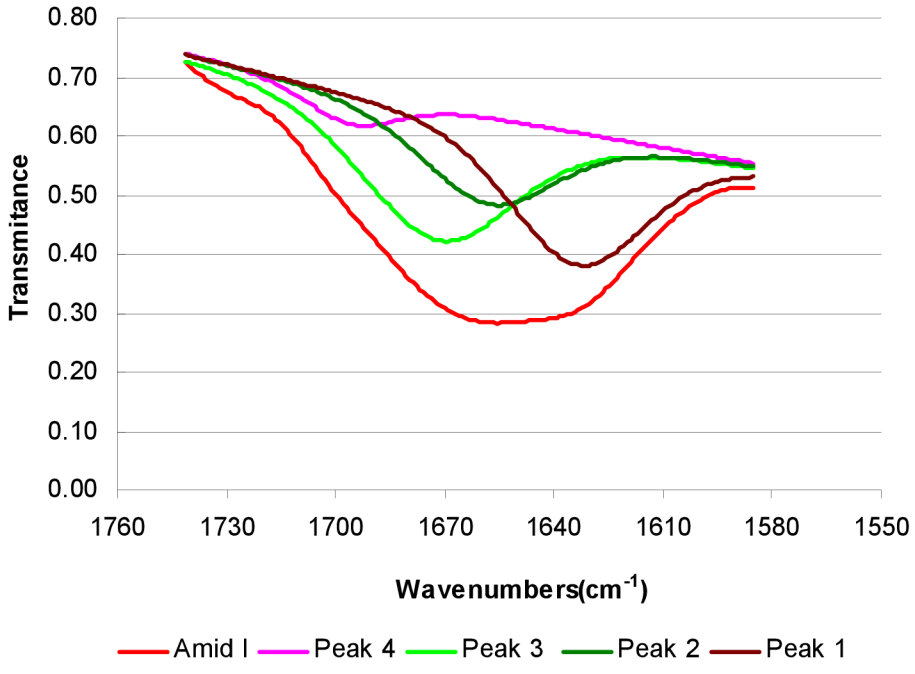
Ratio	Peak 1		Peak 2	
	Peak location [cm^{-1}]	Area	Peak location [cm^{-1}]	Area
1:0	1634,71	7,91	1651,78	5,93
9:1	1632,97	11,08	1657,38	6,78
3:1	1630,06	16,03	1649,41	8,85
5:3	1632,98	20,46	1649,09	9,17
1:1	1626,49	0,094	1641,37	24,68
1:2	1628,97	17,26	1642,48	13,35
1:3	1611,47	7,55	1634,00	14,16
1:9	1607,04	21,79	1634,22	44,80

A 11 Location and percent area contribution of amid I components for the *activated collagen/cellulose mixtures* in situ (peaks 3 and 4).

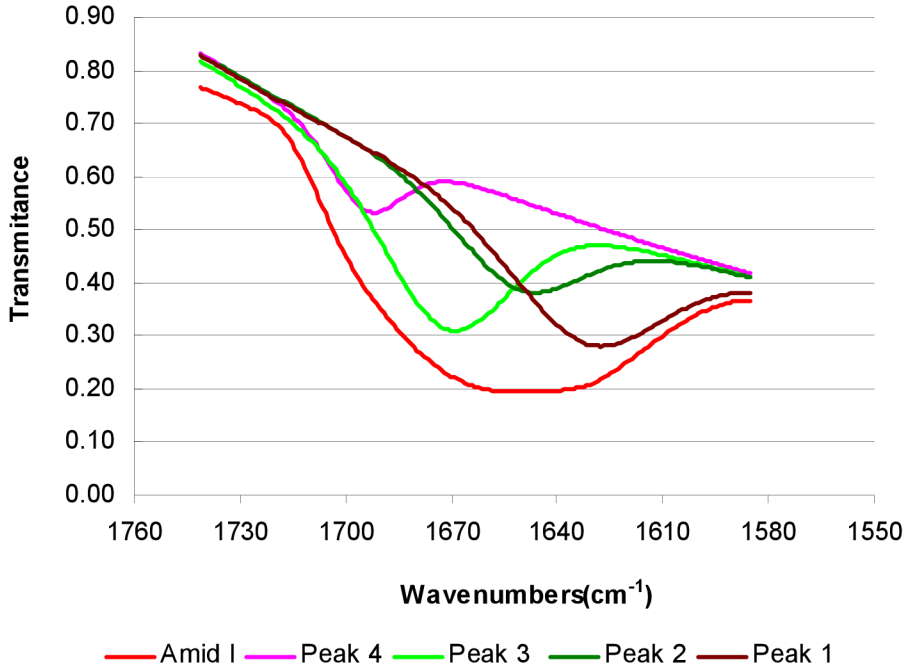
Ratio	Peak 3		Peak 4	
	Peak location [cm^{-1}]	Area	Peak location [cm^{-1}]	Area
1:0	1666,37	13,68	1691,65	2,23
9:1	1670,98	11,82	1695,21	1,77
3:1	1670,38	15,08	1693,79	3,28
5:3	1667,70	21,90	1690,75	4,50
1:1	1669,24	15,16	1698,76	0,00
1:2	1665,29	27,98	1690,26	4,13
1:3	1661,98	38,68	1693,70	2,17
1:9	1664,56	55,25	1692,46	4,47



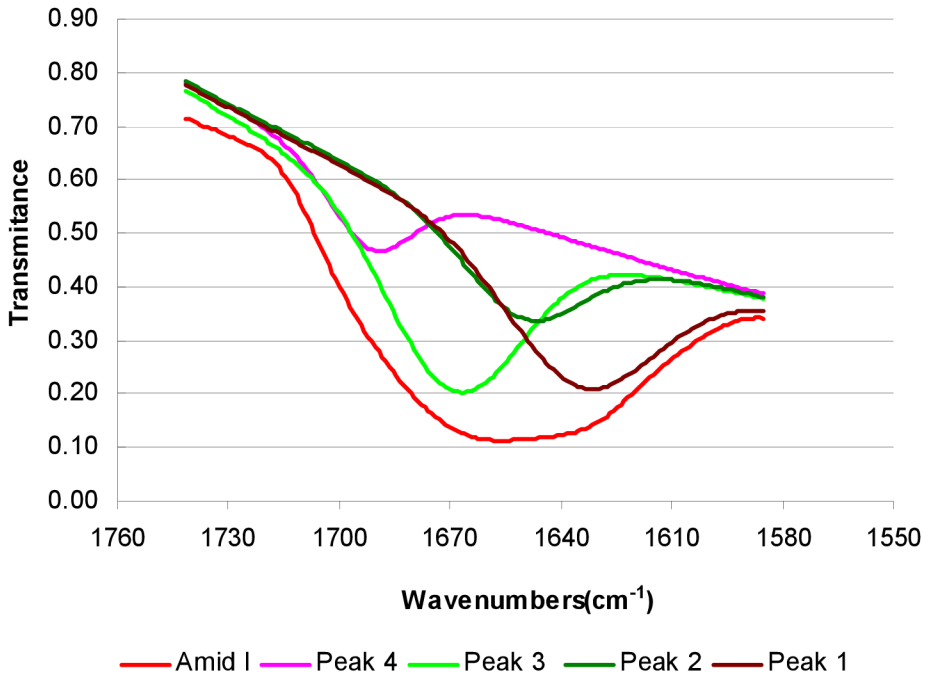
A 12 *Amid I band of activated collagen/cellulose mixtures in situ in the ratio of 1:0 with fitted components.*



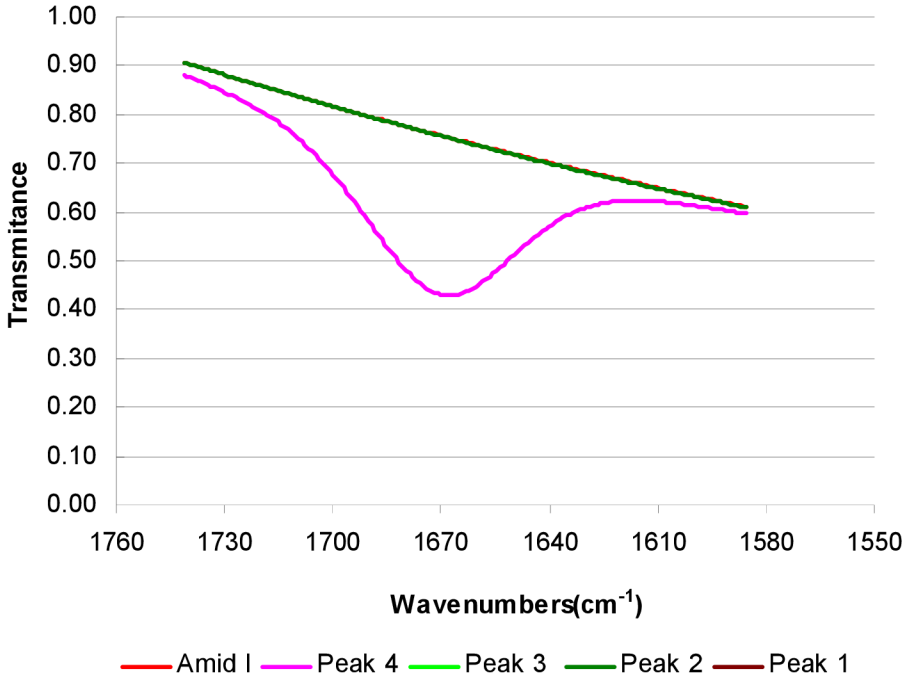
A 13 *Amid I band of activated collagen/cellulose mixtures in situ in the ratio of 9:1with fitted components.*



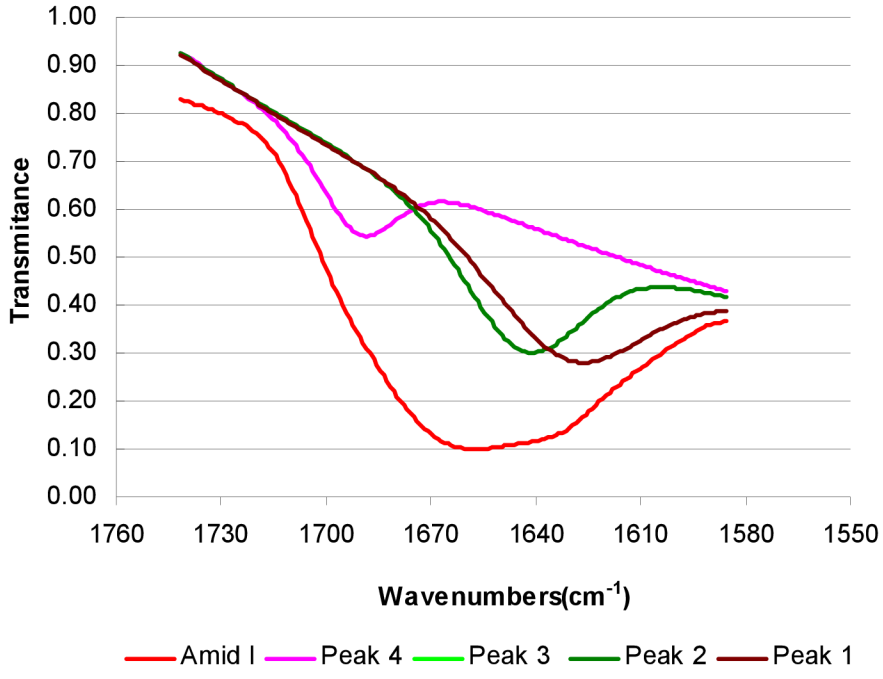
A 14 *Amid I band of* activated collagen/cellulose mixtures in situ *() in situ in the ratio of 3:1with fitted components*.



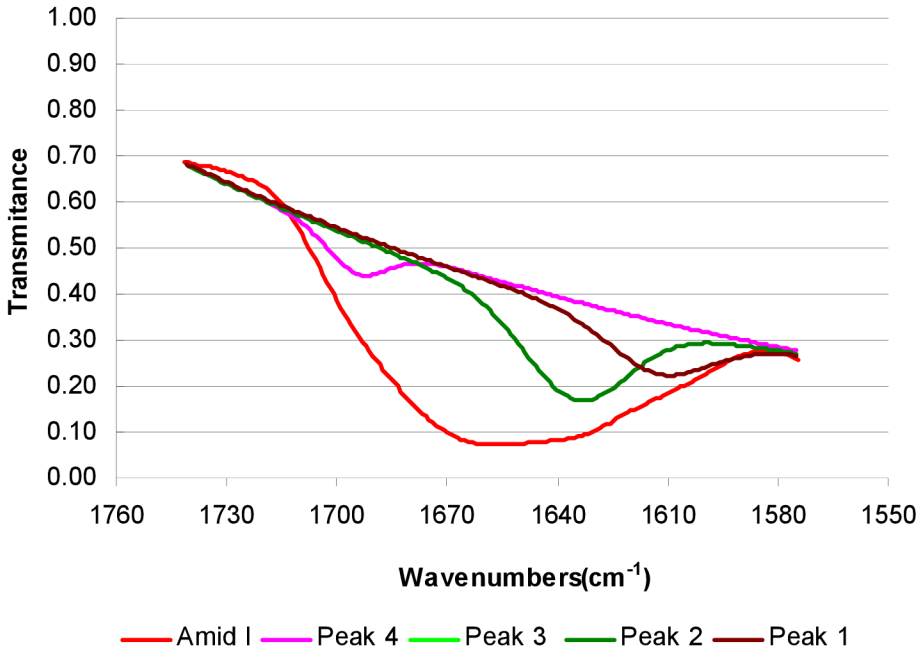
A 15 *Amid I band of activated collagen/cellulose mixtures in situ in the ratio of 5:3with fitted components.*



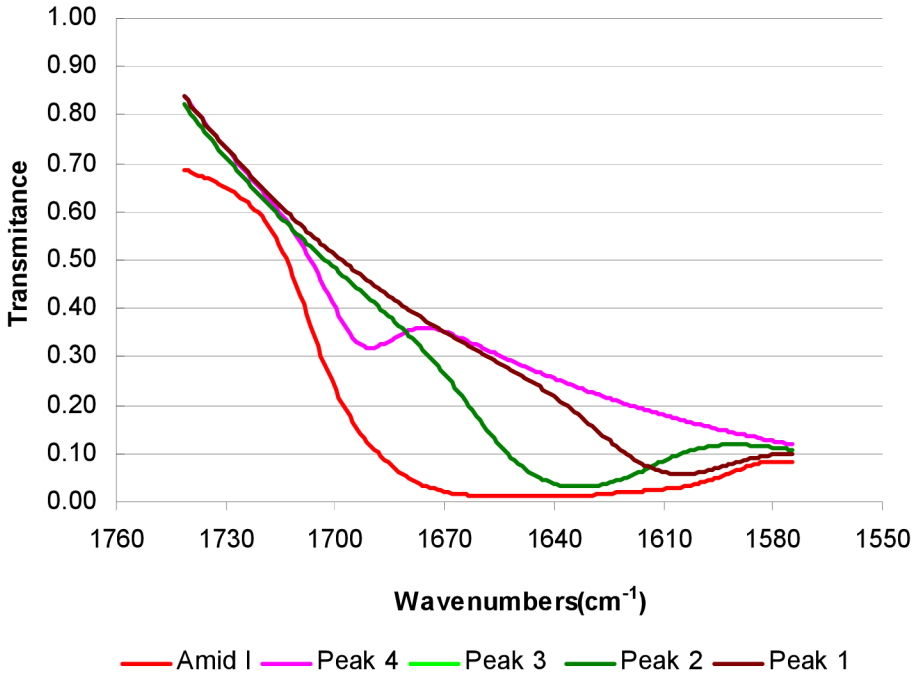
A 16 *Amid I band of activated collagen/cellulose mixtures in situ with in the ratio of 1:1 fitted components.*



A 17 *Amid I band of activated collagen/cellulose mixtures* in situ *in the ratio of 1:2 with fitted components.*



A 18 *Amid I band of activated collagen/cellulose mixtures* in situ *in the ratio of 1:3 with fitted components.*



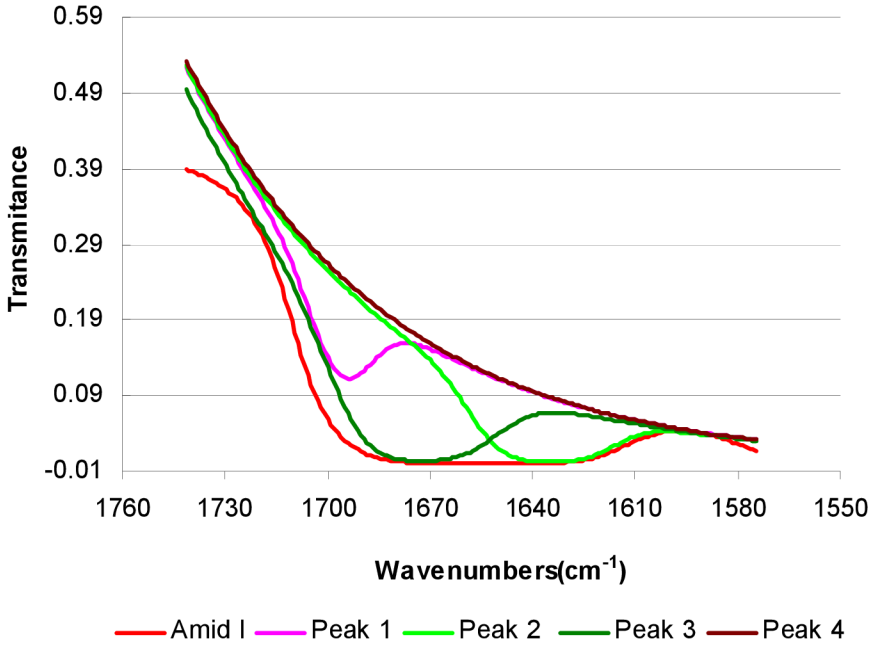
A 19 *Amid I band of activated collagen/cellulose mixtures* in situ *in the ratio of 1:9 with fitted components.*

A 20 Location and percent area contribution of amid I components for the *activated collagen/cellulose mixtures* in the film (peaks 1 and 2).

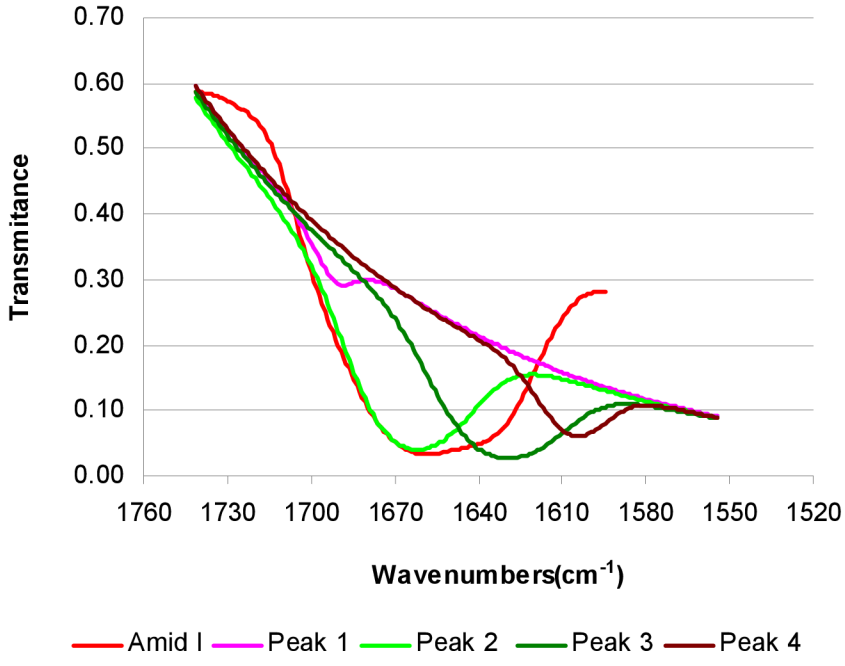
Ratio	Peak 1		Peak 2	
	Peak location [cm^{-1}]	Area	Peak location [cm^{-1}]	Area
1:0	1635.57	51.57	1656.26	45.84
9:1	1634.40	20.50	1652.56	21.21
3:1	1633.02	14.94	1652.23	10.25
5:3	163409	12.88	1657.04	9.50
1:1	1637.11	31.11	1660.35	13.39
1:2	1637.32	36.39	1650.02	0.0074
1:3	1636.74	44.37	1659.85	1.90
1:9	1605.43	11.70	1630.67	40.22

A 21 Location and percent area contribution of amid I components for the *activated collagen/cellulose mixtures* in the film (peaks 3 and 4).

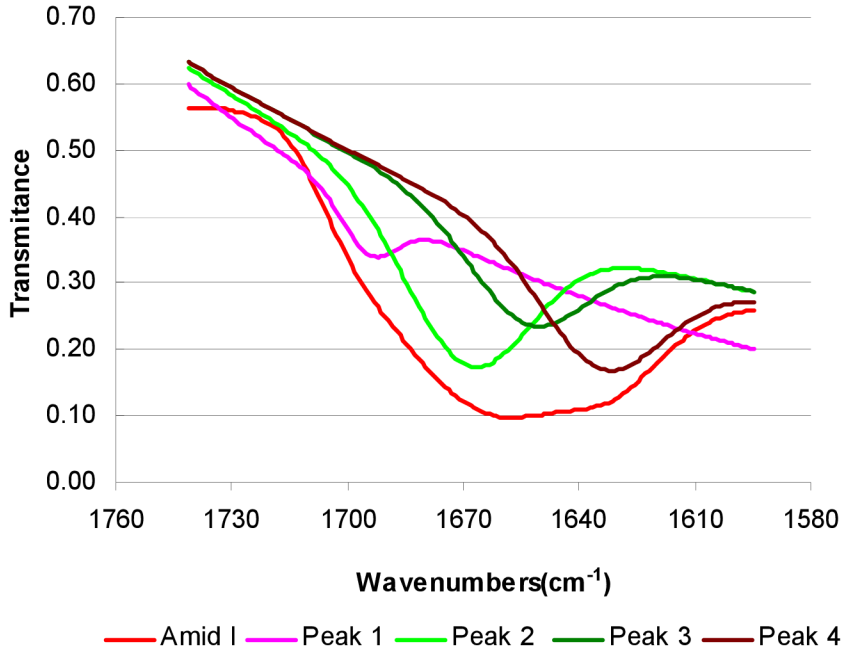
Ratio	Peak 3		Peak 4	
	Peak location [cm^{-1}]	Area	Peak location [cm^{-1}]	Area
1:0	1673.10	67.61	1695.11	8.35
9:1	1666.40	25.56	1688.79	7.08
3:1	1667.59	17.75	1690.43	4.28
5:3	1670.02	9.16	1690.56	1.60
1:1	1675.94	9.78	1693.75	2.03
1:2	1664.72	23.15	1690.46	1.81
1:3	1665.56	27.34	1693.96	1.86
1:9	1662.97	37.54	1691.10	1.97



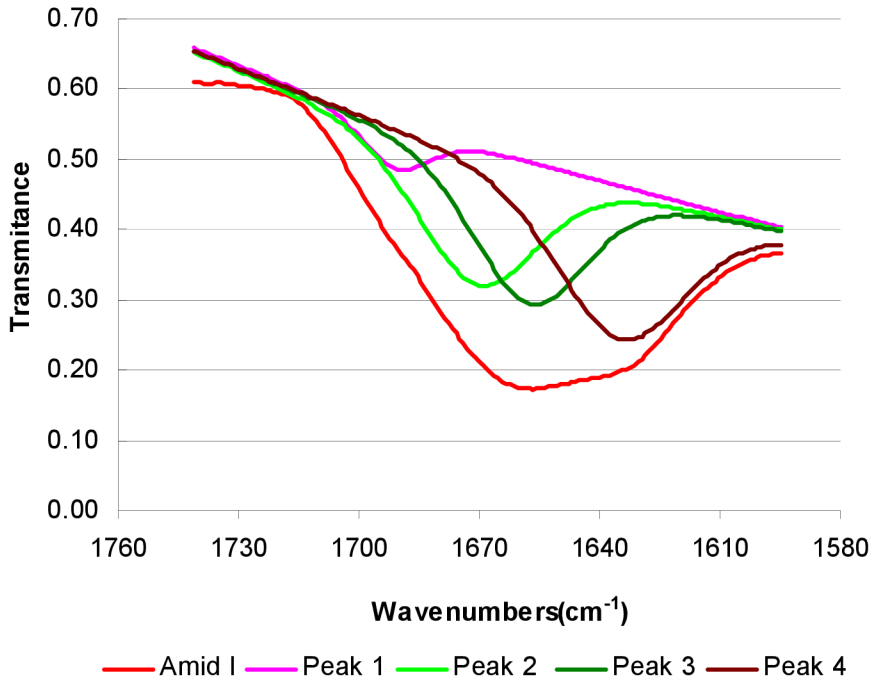
A 22 *Amid I band of activated collagen/cellulose mixtures* in the film *in the ratio of 1:0 with fitted components.*



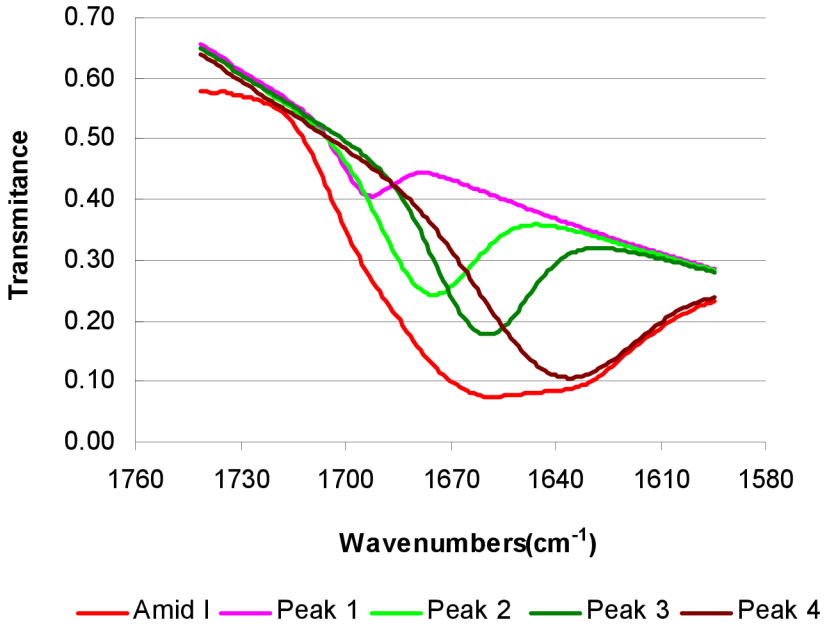
A 23 *Amid I band of activated collagen/cellulose mixtures* in the film *in the ratio of 9:1with fitted components.*



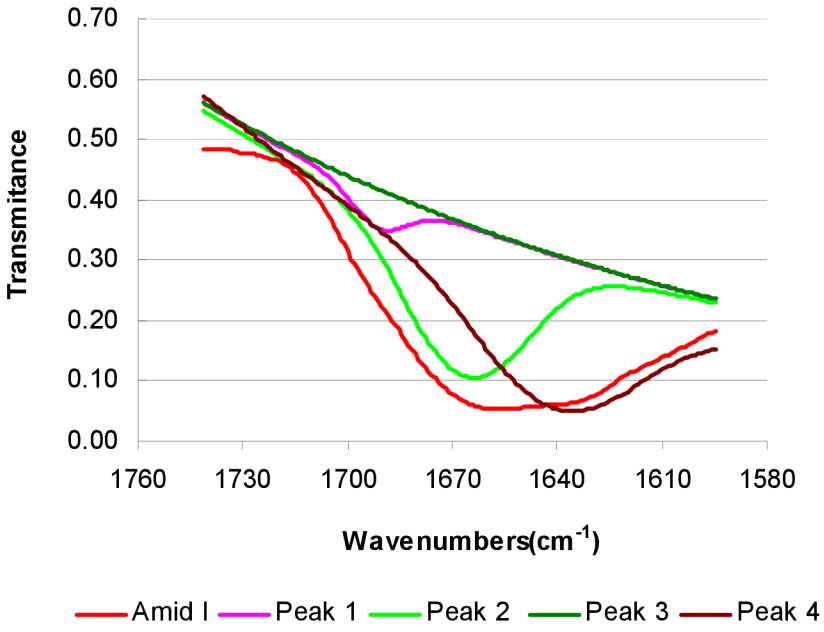
A 24 *Amid I band of activated collagen/cellulose mixtures* in the film *in the ratio of 3:1with fitted components*.



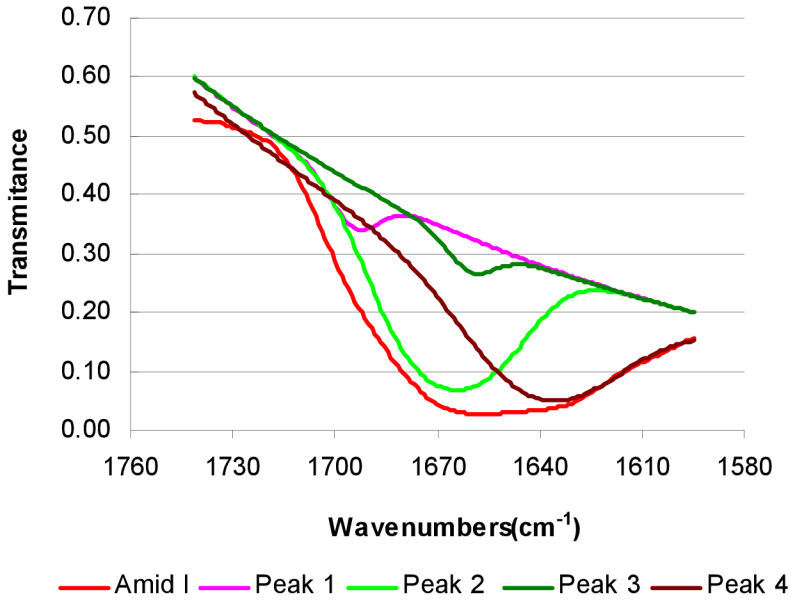
A 25 *Amid I band of activated collagen/cellulose mixtures* in the film *in the ratio of 5:3 with fitted components*.



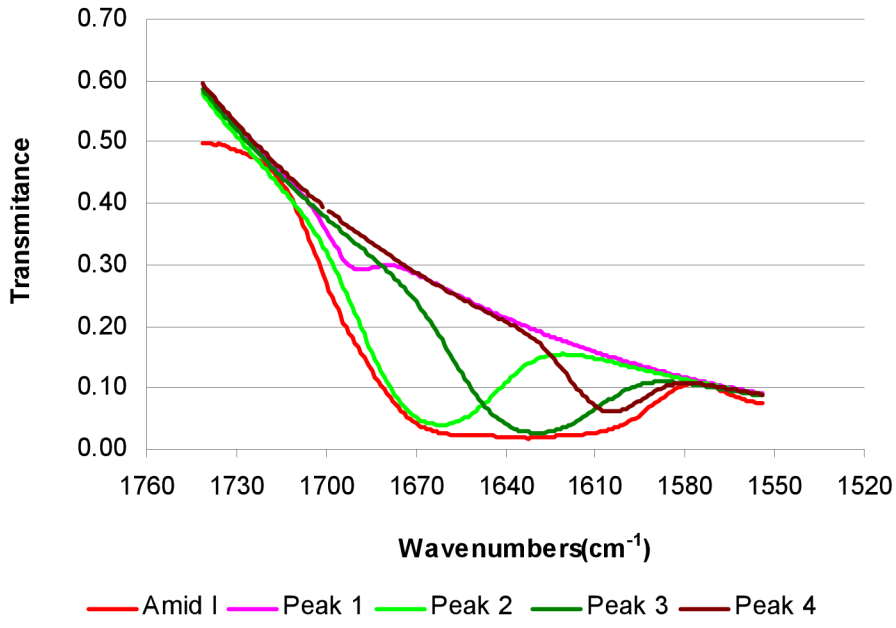
A 26 *Amid I band of activated collagen/cellulose mixtures* in the film *in the ratio of 1:1 with fitted components.*



A 27 *Amid I band of activated collagen/cellulose mixtures in the film in the ratio of 1:2 with fitted components.*



A 28 *Amid I band of activated collagen/cellulose mixtures* in the film *in the ratio of 1:3 with fitted components.*



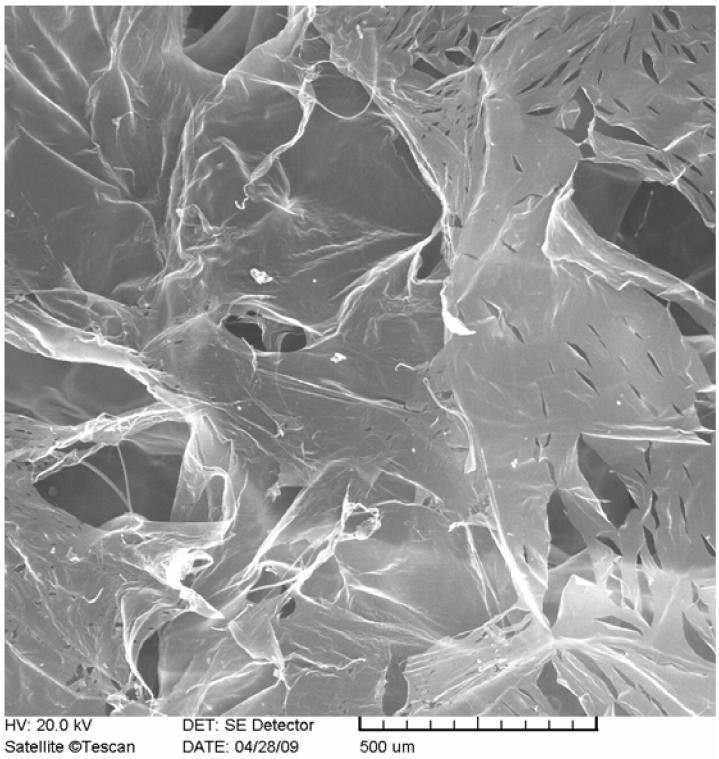
A 29 *Amid I band of activated collagen/cellulose mixtures* in the film *() in the ratio of 1:9 with fitted components.*

A 30 Porosity and pore size of collagen/cellulose mixtures frozen using liquid nitrogen.

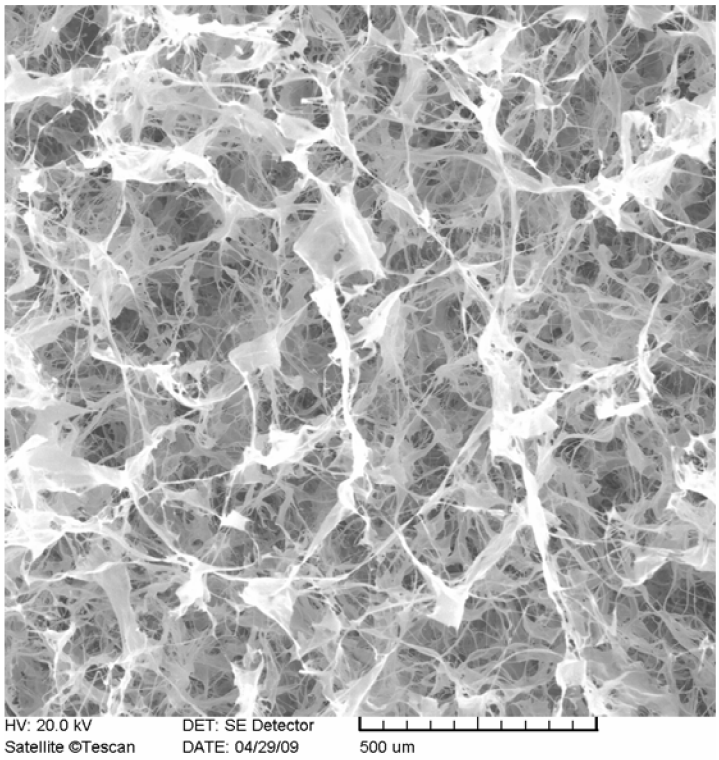
Ratio collagen/cellulose	Porosity (%)	Pore size (μm)
1:0	57.67	13.73 ± 5.57
9:1	59.58	47.11 ± 23.05
3:1	46.70	53.59 ± 30.15
5:3	49.34	50.23 ± 2.50
1:1	46.95	57.72 ± 41.94
1:2	33.81	13.50 ± 9.51
1:3	63.07	54.51 ± 28.92
1:9	80.31	186.13 ± 76.02
0:1	68.39	79.08 ± 24.12

A 31 Porosity and pore size of collagen/cellulose mixtures frozen in the freezer.

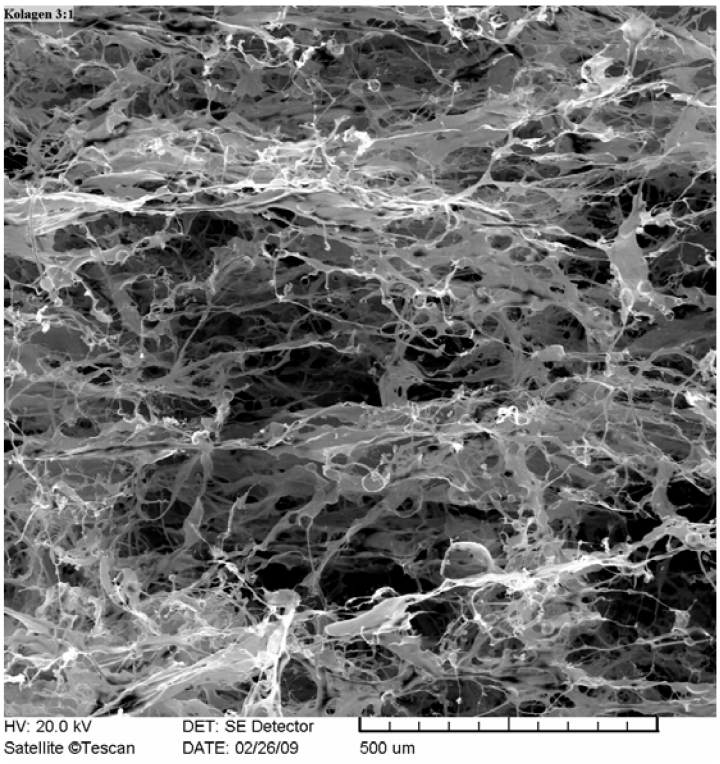
Ratio collagen/cellulose	Porosity (%)	Pore size (μm)
9:1	54.25	57.29 ± 25.49
3:1	46.41	65.92 ± 33.85
5:3	49.28	98.18 ± 60.34
1:1	56.65	41.48 ± 19.52
1:3	62.48	86.80 ± 47.56
1:9	63.72	57.29 ± 25.49



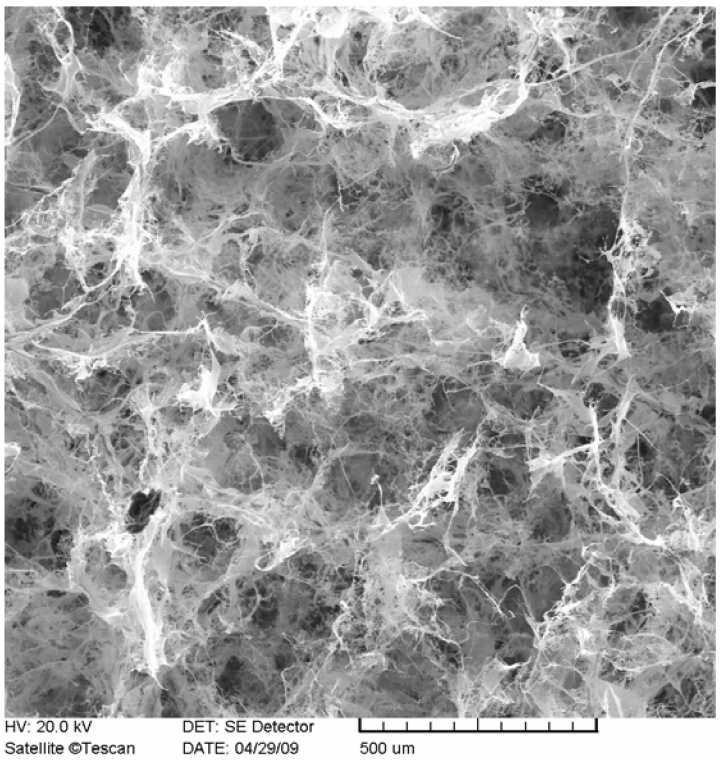
A 32 *Morphology of the ratio collagen/cellulose of 1:0 observed by SEM and frozen using liquid nitrogen.*



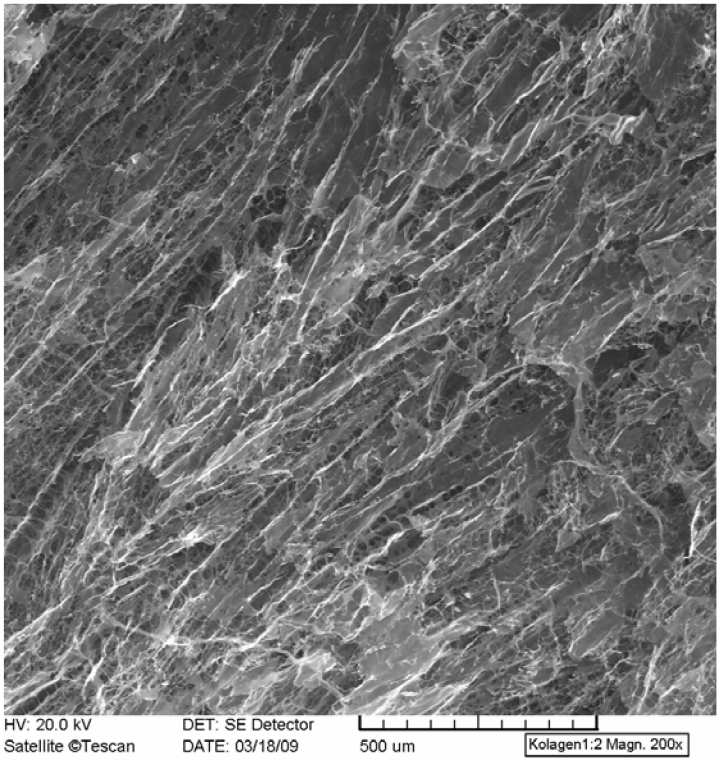
A 33 *Morphology of the ratio collagen/cellulose of 9:1 observed by SEM and frozen using liquid nitrogen.*



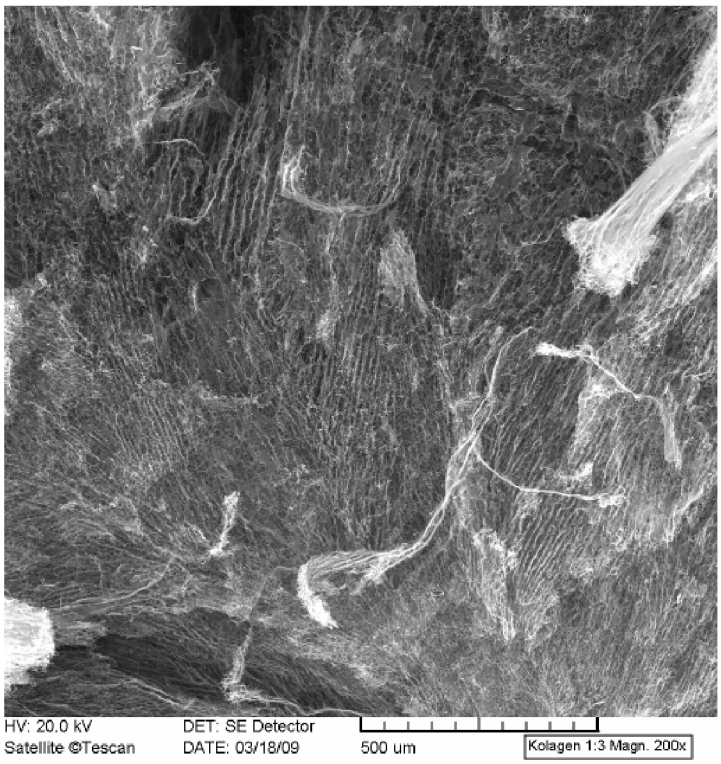
A 34 *Morphology of the ratio collagen/cellulose of 3:1 observed by SEM and frozen using liquid nitrogen.*



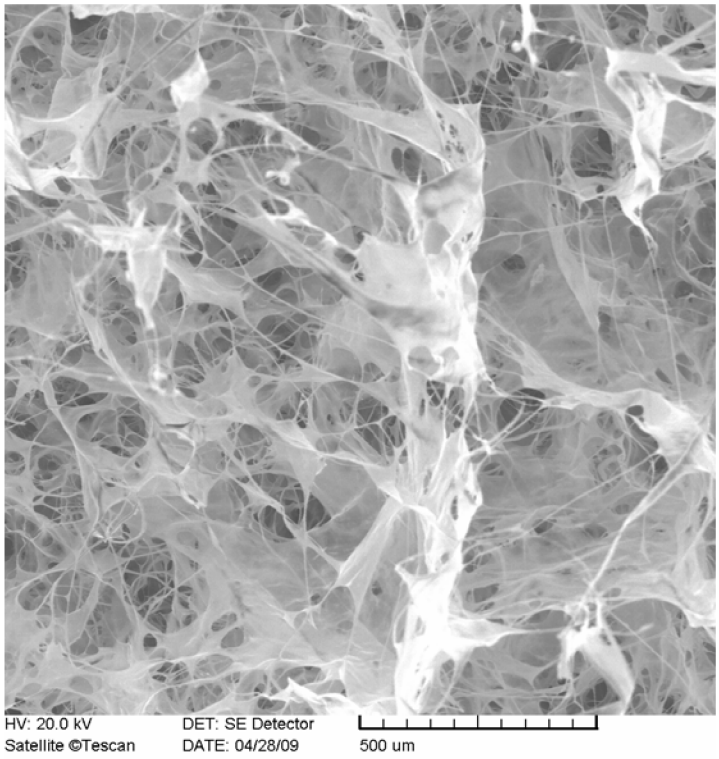
A 35 *Morphology of the ratio collagen/cellulose of 5:3 observed by SEM and frozen using liquid nitrogen.*



A 36 *Morphology of the ratio collagen/cellulose of 1:2 observed by SEM and frozen using liquid nitrogen.*

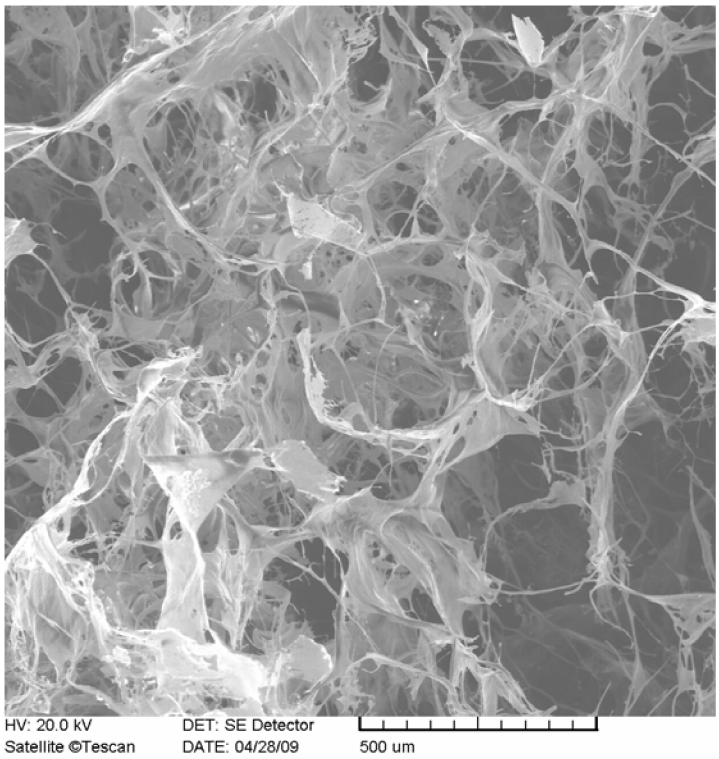


A 37 *Morphology of the ratio collagen/cellulose of 1:3 observed by SEM and frozen using liquid nitrogen.*

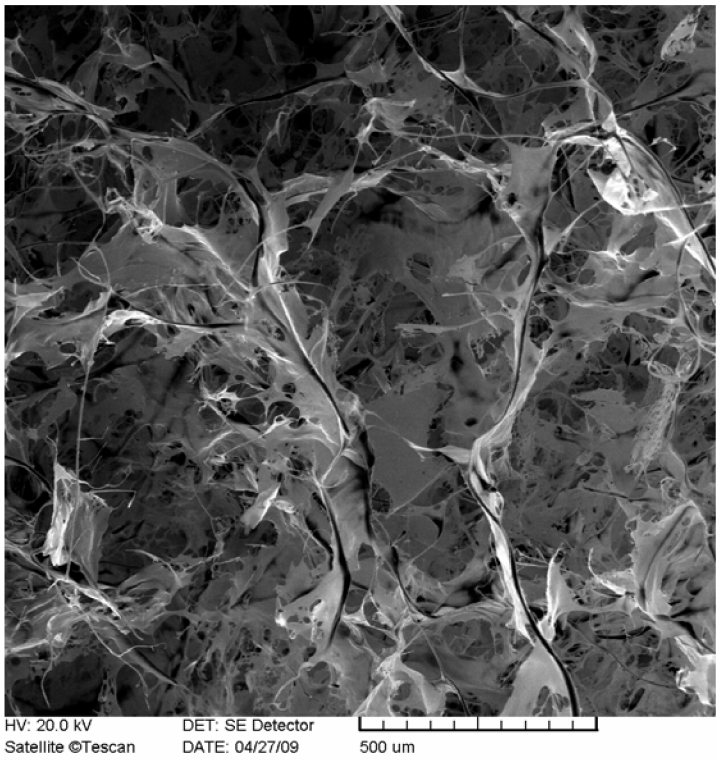


A 38 *Morphology of the ratio collagen/cellulose of 9:1observed by SEM and frozen in the freezer.*

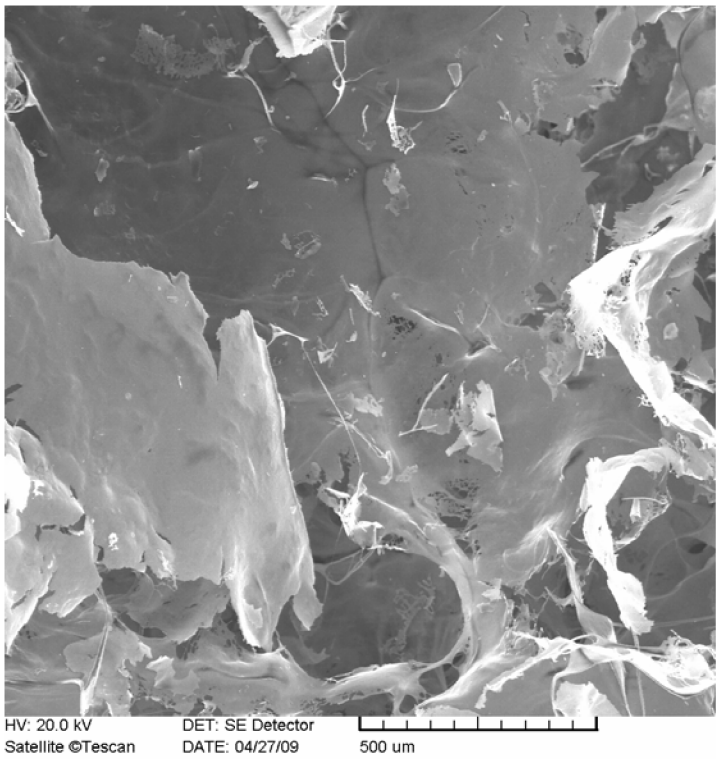
A 39 *Morphology of the ratio collagen/cellulose of 3:1 observed by SEM and frozen in the freezer.*



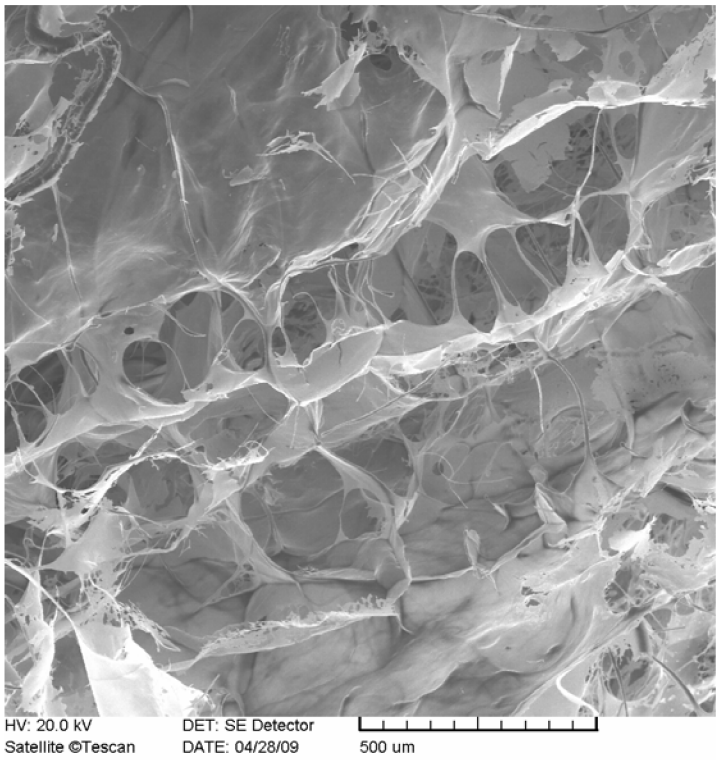
A 40 *Morphology of the ratio collagen/cellulose of 5:3 observed by SEM and frozen in the freezer.*



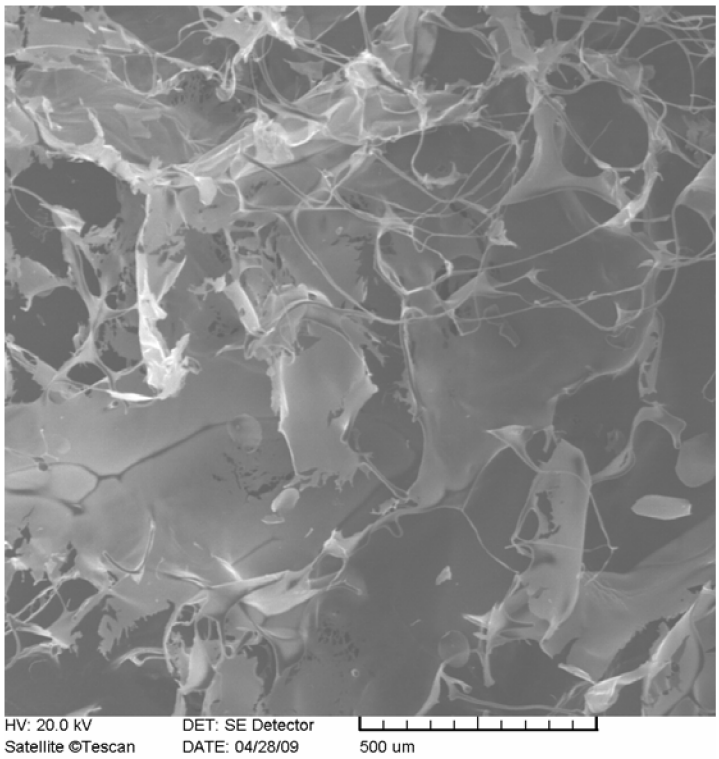
A 41 *Morphology of the ratio collagen/cellulose of 1:1 observed by SEM and frozen in the freezer.*



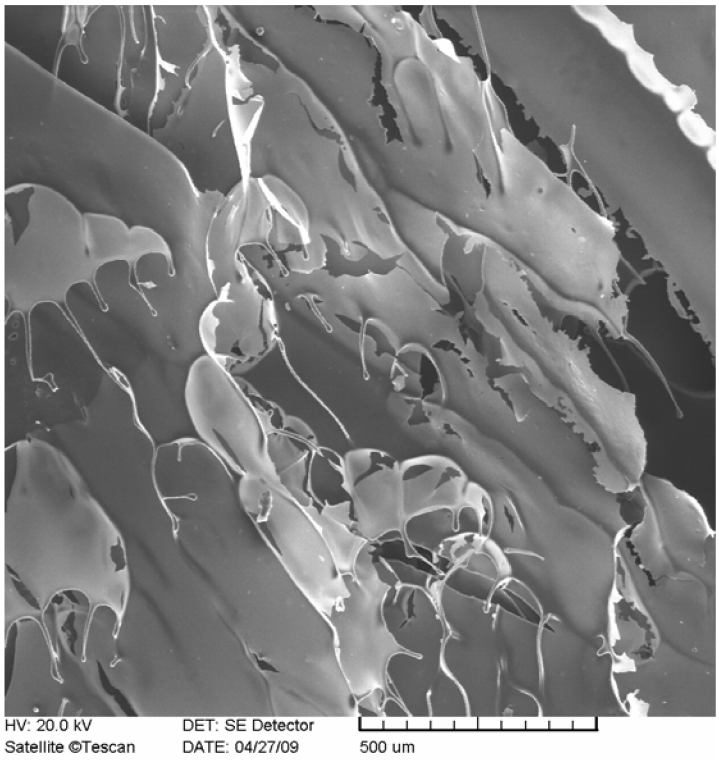
A 42 *Morphology of the ratio collagen/cellulose of 1:2 observed by SEM and frozen in the freezer.*



A 43 *Morphology of the ratio collagen/cellulose of 1:3 observed by SEM and frozen in the freezer.*



A 44 *Morphology of the ratio collagen/cellulose of 1:9 observed by SEM and frozen in the freezer.*



A 45 *Morphology of the ratio collagen/cellulose of 0:1 observed by SEM and frozen in the freezer.*