Improving the Water Resistance of Biodegradable Collagen Films

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ABSTRACT: Although collagen-based films have been successfully used for packaging in the meat industry, their potential as a replacement for synthetic packaging films in other industries has not yet been widely investigated. This may be due to the low water resistance of protein films. The objective of this study was to systematically improve water resistance in collagen-based films and to investigate the influence of different crosslinking agents and crosslinker concentration levels. In this study, the film's water resistance was determined gravimetrically as well as by applying the Sircol™ Protocol. Although the reference collagen film produced without any crosslinking agents showed to have almost completely disintegrated after 2 h at 80°C, it was possible to generate chemically crosslinked films, which stayed intact after 2 h at 80°C and even maintained water resistance after 8 h at 60°C. The results of this study showed that thermal crosslinking leads to weaker bonds than the chemically crosslinking. Both assay methods for the determination of the water resistance yielded almost identical curves, except for films with an added plasticizer, clarifying that the Sircol™ protocol is not suitable to record data as a result of the dissipation of the plasticizer. Furthermore, study results indicated that the water resistance strongly depends on the amount of added crosslinker and reaches a maximum at a concentration of 10% w/w, whereas compostability was nearly 90% at 58°C within 38 days for a chemically crosslinked collagen film plasticized with lecithin. However, increased crosslinking significantly decreased the enzymatic degradability of the investigated films. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: collagen; films; water resistance; crosslinking; biodegradable

INTRODUCTION

Although in the future it may not be possible to entirely replace petroleum-based plastics with bioplastics, biopolymers have shown the potential to reduce the consumption of synthetically produced plastics in certain areas of application.[1] For manufacturing biopolymers, plant- or animal-based polysaccharides, proteins and lipids as well as combinations thereof can be used.[2] Currently, the food industry uses collagen-based films and casings as a standard. Edible collagen films represent the commercially most successful protein films.[3,4] It is estimated that ~ 80% of all produced sausage casings are made from tubular collagen films.[5]

The raw material collagen is a natural and renewable resource,[6] which can be obtained, for example, as a byproduct from leather production[7] and is therefore readily available.[6] Furthermore, collagen is highly biodegradable as opposed to petroleum-based plastics, which are biologically inert for many years or even decades and therefore are not or are only partially compostable.[8] In addition, collagen as biomaterial is physiologically harmless and approved for the food industry.[6] Films and coatings can be manufactured by casting, spraying, or extruding.[9] The fibrous structure of collagen films is responsible for their beneficial mechanical properties.[4]

However, films made from collagen as well as other proteins show a high affinity to water.[6] In contact with water, collagen chemically absorbs water in the form of bound water and partly as capillary water,[1] which alters the properties of the film and at worst, can lead to destruction of the film altogether.[9] Alkaline amino groups and hydroxyl groups as well as carboxyl groups have been shown to be responsible for the high degree of hydrophilicity.[10]

Studies exist regarding the effects of water absorption on mechanical properties[11] as well as studies that determine water solubility[5] of collagen films, but a subject literature search shows the absence of research on how to systematically improve water resistance of collagen films. One study, Pukhova et al.[12] publicized research done on increasing water resistance of collagen fibers during the spinning

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process by the use of Cr_2O_3. Literature search also showed a remarkable absence of consistent terminology. In similar context, different terms are used such as water resistance,[1,13] water solubility,[14–16] water absorption,[10,12] water repellence,[14] or water permeability[13,17–19] but often not clearly defined. This study defines water resistance as the film's ability to resist dissolution in contact with water or to dissolve to a certain degree only.

Based on the fact that proteins, thus collagens accordingly, consist of varying amino acids with different side chains, it has been shown possible to modify the properties of protein films through chemical modification of their side chains. The inter- and intramolecular crosslinking with aldehydes, such as glyoxal,[2] formaldehyde,[5,16] and glutaraldehyde,[3,5,16] respectively, as well as carbodiimide[4] shows positive effects[3,2,9] regarding water resistance[5,15,20,21] by forming a water-insoluble three-dimensional network via covalent bridges between protein chains. By comparison, physical treatments, such as UV[3]- or dehydrothermal (DHT)crosslinking[5] lead to crosslinked proteins, however, with significantly less stable bonds.[22]

With respect to the use of protein films, other than water resistance, attention also needs to be paid to the brittleness, hence the low degree of elastic plasticity of these films.[9] Plasticizers decrease brittleness by decreasing intermolecular forces between polymer coils, which leads to the loss of density of the protein scaffolding[9,19] and thus to an increase in material flexibility. However, with increasing levels of plasticizer content, permeability is increased subsequently due to the augmentation of the free volume/intermolecular spacing,[9] which is why great detail has to be given to this aspect with regards to investigating water resistance.[9,19] Furthermore, most plasticizers are hygroscopic in nature, which hence increase the water content of films.[23]

Finally, an important reason for using renewable resources is their biodegradability. Degradability in this context is to be understood as enzymatic degradability as well as compostability. Increasing water resistance of collagen films by chemical crosslinking may alter degradability and compostability, therefore, it is important to pay attention to these two aspects.

Motivation for this study was the realization that it is most likely the nonbeneficial effect of water on the properties of protein films that is responsible for the limited use of the full potential of collagen films outside the meat processing industry. The object of this study was to systematically examine how to improve water resistance of collagen films. Therefore, a step-by-step approach was chosen for the optimization. Figure 1 shows the individual steps during the optimization process. At first, it was examined whether

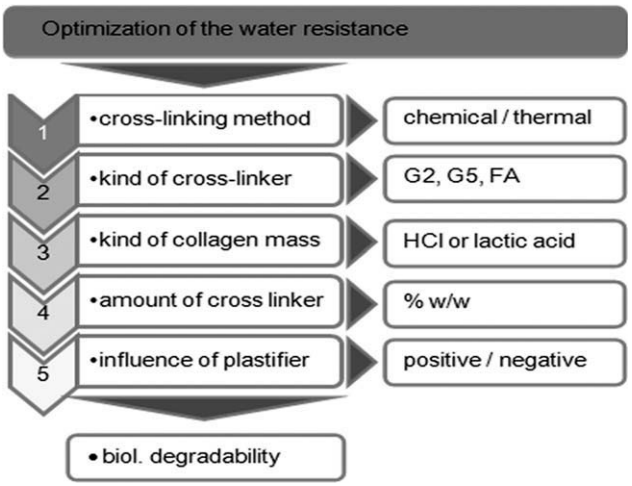

Figure 1 Procedure for optimization of water resistance.

thermal or chemical crosslinking produces more water resistant films. Afterward, three different crosslinking agents and combinations thereof were compared with regards to their capability to produce the most water resistant film. Subsequently, it was tested to what extent the behavior of a collagen suspension acidified with hydrochloric acid (collagen suspension A) would differ from a mass acidified with lactic acid (collagen suspension B). As above, the better result was selected for further testing and the appropriate crosslinker concentration was ascertained. Afterward, the influence of the plasticizer on water resistance was determined. Finally, it was assessed whether the improved water resistance of collagen films negatively affected their compostability.

EXPERIMENTAL

Materials and reagents

Unless specified otherwise, all chemicals were obtained in analytical grade from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), or VWR (Darmstadt, Germany). Collagen film Cobiosh at experimental stage (thermally crosslinked, 7401-4) and hydrochloric acid (collagen compound A) as well as lactic acid (collagen compound B) prepared from a bovine connective tissue collagen matrix with 8.6% (12%) dry collagen content, was provided by Naturin Viscofan GmbH (Weinheim, Germany). Ethanedial (glyoxal, G2) of 40% in aqueous solution for synthesis was purchased from Merck (Darmstadt, Germany). Methanal GPR Rectapur (Formaldehyde, FA) 36% was purchased from VWR (Darmstadt, Germany) and Pentane-1.5-dial(glutaraldehyde, G5) was provided by Naturin Viscofan GmbH (Weinheim, Germany). Aluminum sulfate hydrate crystals and lecithin >97% for the biochemistry was obtained from Roth (Karlsruhe, Germany). Methyl 3-hydroxybenzoate 99%,

trans, trans-2.4-acid potassium salt hexadienoic (99%) and ethyl 4-hydroxybenzoate 99% as a preservative were obtained from Aldrich (Steinheim, Germany). Deionized water was obtained from an in-house supply. Pronase from *Streptomyces griseus* with an activity 4,000,000, PU/g was obtained from VWR (Darmstadt, Germany). Trypsin (2500 USP-U/mg) was purchased from VWR (Darmstadt, Germany), protease A-01 and A-08 (500 U/mg or mL) was purchased from ASA Spezialenzyme (Wolfenbüttel, Germany). Phosphate buffered saline (PBS) BDH Prolabo, pH 7.2 was purchased from VWR (Darmstadt, Germany).

OxiTop with MG 1.0 bottle for soil respiration determination were obtained from WTW Wissenschaftlich Technische Werkstätten (Weinheim, Germany). TESA Polyolefinfolie Bodyguard® 50530 PV7 (with EVA polyethylene-adhesive layer) was obtained from FN Klebe und Schleifprodukte GmbH (Ergolding, Germany). Compost with a grain size of 1–10 mm was purchased from a local composting plant, Wagner GmbH (Frankenthal, Germany). Cellulose for thin layer chromatography (< 20 μm) was obtained from Aldrich (Steinheim, Germany).

Preparation of chemically crosslinked collagen films

Unless otherwise stated, all concentration specifications are provided in % w/w (weight portion) and are referring to the collagen dry matter content. For the production of 350 g collagen suspension with a dry collagen fraction of 2.5% (unless indicated otherwise), 73 g (91.5 g) with a meat grinder minced collagen suspension A (collagen suspension B) were added to 274 g aluminum sulfate[24] solution (0.015% w/w) and homogenized on Level 2 for 20 min with homogenizer (Ultraturrax T50 Basic, Ika-Werke, Staufen, Germany). Aluminum sulfate was used following a recipe of Naturin Viscofan GmbH (Weinheim, Germany). It reduces the viscosity of the collagen suspension through reduction of the hydration shell (reduces swelling) and therefore enables the use of higher collagen contents, which further minimize the drying time.[24] The collagen was left to soak overnight. The following day, additives such as plasticizers were added to the collagen suspensions according to test requirements (see corresponding experimental section for more details). After mixing all additives, the pH value was adjusted to 2.8 with 90% lactic acid or hydrochloric acid. The resulting mass was homogenized with the homogenizer on Level 2 for an additional 30–45 min. To produce a film, it was proven to be necessary to work with a level of 3 mm filled into the casting molds (Teflon dishes, d =13 cm, VWR, Darmstadt, Germany or PP wells, manufacturer unknown) and mixed with (if not stated otherwise) 10 g of a crosslinker solution (corresponds to 10% w/w) and further degasified in a vacuum cabinet (WTB Binder GmbH, Tuttlingen, Germany) at 5 mbar for 20 min. The degassed compounds were dried overnight in a warm-air compartment dryer (custom built). Figure 4 shows the finished film. As reference film, a collagen film manufactured out of a collagen suspension without the addition of crosslinkers was used.

Preparation of thermally crosslinked collagen films

The thermally crosslinked collagen film (Cobiosh 7401-4) was provided by Naturin Viscofan GmbH (Weinheim, Germany). The film was prepared from a bovine collagen (Type I) through extrusion. After drying at 50°C for 24 h in a circulating air oven, the film was DHT crosslinked at 105°C for about 1 h.

Determination of water resistance of the collagen films in accordance with DIN EN ISO 175

The water resistance of the test films was determined in accordance with DIN EN ISO 175,[25] Plastics determination of the effects of immersion in liquid chemicals. Diverging from DIN EN ISO 175 for the test 100 mg of collagen film (see corresponding experimental section for more detailed information regarding amount of crosslinkers) was incubated in 8 mL deionized water for 1 h and 2 h, respectively (see experimental section), at 20°C, 40°C, 60°C, and 80°C. At the end of the test period, undissolved film was separated from the test fluid by centrifugation with a centrifuge (J2-HC, Beckmann Coulter GmbH, Krefeld, Germany) at 10,000 rpm for 2 min, and the dissolved collagen content was determined. For the determination of the dissolved collagen fraction, two methods were used. For one, the spectrometric assay of soluble collagen by using Sirius Red dye reagent according to the Sircol™ Protocol by Biocolor Ireland[26] and furthermore by gravimetrical determination of decrease in weight of the collagen.

Sircol™ protocol

Collagen content in the supernatant was determined according to the Sircol™ Protocol by Biocolor Ireland.[26] Instead of dissolving the dye in pipric acid, deionized water was used according to Lee et al.,[27] and the pH was adjusted to 2.0 with 1*M* HCl.[27] The extinction was measured in a photometer (CADAS 200, Hach Lange GmbH, Düsseldorf, Germany).

To determine the collagen concentration of the supernatant, a calibration curve was created. The collagen content of the probes was calculated according to eq. (1), and the water resistance was calculated according to eq. (2).

$$\text{collagen content } c\ [\text{mg}/100\ \text{mL}] = \frac{E[555\ \text{nm}] + 0.0142}{0.0186} \quad (1)$$

where c is the collagen content in mg/100 mL and E is the extinction at 555 nm [ ].

Gravimetric detection

The gravimetric determination of the difference between the collagen suspensions before and after treatment was performed referring to Tint[28] with a Precision Scale (II-150, SNUG Jadever Scale Co., Markham Ontario, Canada). For evaluation, the film test specimens were weighted out precisely to 0.0005 g (m_1) at the beginning of the assay and then weighed again (m_2) after incubation, decanting of test fluid, rinsing, and drying. After quintuple determination, the average value was calculated out of individual measurements.

Water resistance r was determined as follows [eq. (2)]

$$\text{water resistance } r = m_2/m_1 * 100 \tag{2}$$

where r is the water resistance in [%] and m_1 and m_2 are the weights at the beginning, respectively, at the end.

Compostability

Aerobic biodegradability of the collagen films was investigated following the DIN EN ISO 14855-1,[29] 2007 protocol (Regulations for thorough aerobic biodegradability of plastic materials under controlled composting conditions), with the polyolefin film as reference material. After adjusting the water content of the compost with tap water, the dry matter content was determined at 49.73% with a moisture analyzer (MA 35, Sartorius, Göttingen, Germany). The ash, or respectively, the organic dry substance was determined with an incinerator (ULTRA X 05, a&p Instruments, Detmold, Germany) at 3.05/10 g and 1.93/10 g. Glass jars were used as composting containers with an inserted metal grille so that the compost could be ventilated evenly through a hose attached at the bottom. The ventilation was performed with CO_2-free air (which was produced via induction into a 4*M* sodium hydroxide-filled wash bottle as a trap. Diverging from DIN EN ISO 14855-1 first, 100 g of the wetted compost was placed onto the ventilation grille of each container, then each of the test films were placed onto the compost and finally covered with another layer of 100 g of compost and sealed with a lid. The collagen films used in the composting experiments were prepared from the collagen suspension B. The suspensions contained 2.5% dry-collagen, 20% w/w lecithin, 20% w/w glycerol, 0.015% w/w aluminum sulfate, and 15% w/w gelatin. Diverging from DIN EN ISO 14855-1, the films used had a diameter of 7.5 cm and a thickness of 110–124 μm (80 μm polyolefin). The incubation was performed in a drying cabinet (FT 420 K, Heraeus Holding GmbH, Hanau, Germany) at 58°C ± 2°C in the dark. Once per day, the mixtures were stirred up, the films removed, rinsed with deionized water, gently dried, and their appearance (size, color, swelling, and decomposition) documented.

TABLE I
Information on the Soil

Parameter	Value
Soil type	Compost
Soil grain size	1–10 mm
Soil density	2 kg/L
Soil volume	50 mL/100 g

Due to the fact that the experiments described above only prove disintegration, which can be the result of dissolution without degradation, the percentage of biodegradation based on the CO_2 production as definite proof of composting was investigated in parallel. Therefore, 1 L glass vessels were filled with 100 g of the prepared compost (49.73% dry matter content). Table I shows some characteristic information on the soil and the testing parameters.

The collagen films used in these experiments were prepared from the collagen suspension B. The lecithin film contained 2.5% dry-collagen, 20% w/w lecithin, 20% w/w glycerol, 0.015% w/w aluminum sulfate, and 15% w/w gelatin. The noncrosslinked film was prepared with the collagen suspension B (2.5% dry-collagen). Furthermore, the Cobiosh film (thermally crosslinked film) was investigated (for detailed information concerning film composition see Table II).

The film samples were positioned in the middle of the soil. With the used sample sizes of 4.2 × 4.2 cm^2 (9.4 × 9.4 cm^2 for the chemically crosslinked film), it was ensured that the oxygen content of the free gas volume of each test device was sufficient to enable a 100% degradation of the films. The theoretical CO_2 content ($ThCO_2$) (see Table II) produced by total oxidation of the material was calculated from[29]

$$ThCO_2 = TS * C_{tot} * \cdot \frac{44}{12} \tag{3}$$

where TS is the total dry matter (g) of the film component in the film, C_{tot} is the proportion of TOC in film component TS (g/g), 44 is the molecular mass of CO_2 (g/mol), and 12 is the atomic mass of C (g/mol).

In each bottle, a beaker containing 100 mL 1*M* NaOH as CO_2-absorber was placed, and the vessels were closed air-tight. The samples were incubated in an incubation cabinet (FT 420 K, Heraeus Holding

TABLE II
Film Composition and $ThCO_2$

Film	Film component	Dry matter content (g)	Molecular formula	C_{tot} (g/g)	$ThCO_2$ (g)	$ThCO_2$ (mol)
Noncrosslinked	Collagen	0.13	$(C_{12}H_{24}N_3O_4)$	0.48	0.225	0.005
Thermically crosslinked	Collagen	0.21	$(C_{12}H_{24}N_3O_4)$	0.48	0.362	0.008
Chemically crosslinked	Collagen	0.10	$(C_{12}H_{24}N_3O_4)$	0.48	0.175	
	Lecithin	0.03	$C_{42}H_{80}NO_8P$	0.67	0.065	
	Glycerin	0.03	$C_3H_8O_3$	0.39	0.038	
	Gelatin	0.02	$(C_{102}H_{151}O_{39}N_{31})$	0.50	0.037	
	Glyoxal	0.01	$C_2H_2O_2$	0.41	0.020	
	Total				0.335	0.008
Cellulose	Cellulose	0.23	$C_{12}H_{22}O_{11}$	0.42	0.350	0.008

GmbH, Hanau, Germany) at a temperature of 58°C ± 2°C. To maintain sufficient aerobic conditions, vessels were flushed every 3–4 days for 10 min with air, and the NaOH was replenished. The produced carbon dioxide was absorbed in the sodium hydroxide solution and determined every 3–4 days by titration with 1*M* HCl to phenolphthalein end point after addition of 20 mL 0.5 mL $BaCL_2$ solution. Quantity of carbon dioxide was calculated according eq. (4).[30]

$$n\mathrm{CO_2} = \frac{1}{2} V_{\mathrm{NaOH}} \times \left(c_{\mathrm{NaOH-init}} - \frac{c_{\mathrm{HCl}} * V_{\mathrm{HCl}}}{V_{\mathrm{NaOH-titr}}} \right) \quad (4)$$

[30]where $n\mathrm{CO_2}$ is the mass quantity of captured CO_2 (mol), V_{NAOH} is the volume of NaOH as absorber (100 mL), $c_{\mathrm{NAOH\text{-}init}}$ is the NaOH concentration in absorber before test (1 mol/L), c_{HCl} is the concentration of HCl solution (1 mol/L), V_{HCl} is the consumed HCl solution in titration (mL), and $V_{\mathrm{NaOH\text{-}titr}}$ is the volume of NaOH absorber for titration (100 mL).

Percentage of biodegradation D_T was calculated from accumulated amount of CO_2 using the following formula (5). Respiration of a compost blank was subtracted from the values of the sample.

$$\mathrm{DT} = \frac{n(\mathrm{CO_2})\text{film sample} - n(\mathrm{CO_2})\text{soil blank}}{n\mathrm{ThCO_2}} \times 100 \quad (5)$$

[30]where D_T is the percentage of biodegradation according to produced CO_2 (%), $n(CO_2)_{\text{film sample}}$ is the accumulated CO_2 quantity released by the film (mol), $n(CO_2)_{\text{soil blank}}$ is the accumulated CO_2 quantity generated from soil (mol), and $n\mathrm{ThCO_2}$ is the level of theoretical CO_2 quantity (mol).

Enzymatic degradability

Enzymatic degradability of collagen samples was tested according to a modified protocol described in the literature by Angele et al.[31] and Junqueira et al.[32] To determine the degradability, 40 g collagen suspension B with a collagen content of 2% were weighed into a 13 cm Teflon dish (VWR, Darmstadt, Germany) and mixed with 8 mL deionized water, respectively, with 8 mL crosslinker solution. The crosslinker solution was prepared by bringing 2.5 g glutaraldehyde (40%), 2 g glyoxal (50%), and 2.7 g formaldehyde (37%) to 100 mL volume with deionized water and another one with only 2.5 g glutaraldehyde (40%) to 100 mL volume with deionized water, respectively (see experimental section). Using 8 mL of this solution on 40 g collagen suspension resulted in a concentration of 10% w/w based on the collagen dry matter content. For the lower crosslinker concentrations, a 1 : 10 and a 1 : 100 diluted solution were each prepared. All collagen suspensions were dried in a warm-air (custom built) at 37°C for a 24 h period. Enzyme solutions with an enzymatic activity of 6 U were prepared in 60 mL PBS buffer and adjusted by addition of 1*M* sodium hydroxide solution (trypsin and pronase → pH 7.5; proteases → pH 10). For the degradability studies, 200 mg of dried collagen (m_1) were weighed into an Erlenmeyer flask, mixed with 60 mL enzyme solution, and incubated at 40°C (proteases, pronase) and 25°C (trypsin), respectively, with the incubator shaker KS4000i (IKA-Werke, Staufen, Germany) at 100 rpm for 2 h. The enzymatic reaction was stopped by cooling to 4°C in a centrifuge (J2-HC, Beckmann Coulter GmbH, Krefeld, Germany) at 10,000 rpm for 10 min during which the undissolved collagen film was separated. Subsequently, the pellet (undegraded collagen film) was washed with deionized water twice, centrifuged once more, and then according to the gravimetrical determination procedure by Tint[28] left to dry overnight in compartment dryer (custom built) at 37°C and weighed (m_2). The degradability was determined (%) according to eq. (6). The decrease in weight of the corresponding film masses that were incubated at 40°C for 2 h in PBS buffer without enzyme addition (blank value)

were subtracted from the determined dry weight of the collagen film after enzymatic degradation.

$$\text{degradability} = \frac{m_1 - m_2}{m_1} * 100 \qquad (6)$$

where d is the degradability in (%) and m_1 and m_2 are the weights at the beginning, respectively, after 2 h (each blank value adjusted).

RESULTS AND DISCUSSION

Influence of crosslinking method on water resistance of collagen films

Water or moisture can change the properties of collagen films and lead to their destruction. Minimal research on how to systematically improve water resistance of collagen films to increase the potential of collagen films outside of the meat processing industry has been published. As there is no universally agreed on definition, the term water resistance is defined for this study as the film's ability to resist dissolution in contact with water or to dissolve to a certain degree only. In this study, a step-by-step approach was chosen for the optimization of the water resistance of collagen films.

At first, it was examined whether thermal or chemical crosslinking produces more water resistant films (see Fig. 1 Step 1), because Lew et al.[33] and Abke[34] reported that thermal crosslinking in comparison with chemical crosslinking leads to proteins, however, with significantly less stable bonds. Therefore, water resistance of a commercially available collagen film (Cobiosh) compared with an untreated collagen film (reference film made from collagen suspension A) was tested as described in the materials and methods section.

Figure 2 shows water resistance of the thermally crosslinked collagen film in comparison with the reference collagen film at different temperatures, measured after 2 h by Sircol™ protocol and gravimetrically. Both assay methods for the determination of the chemical resistance yielded almost identical curves.

Within the testing period, films produced from a collagen suspension without the addition of any crosslinking agents (reference film made from collagen suspension A) were, up to a water temperature of 37°C–40°C, almost 100% water resistant. Initially, the resistance documented showed little change but decreased significantly after exceeding the denaturation temperature of >40°C (Fig. 2).

These findings may be explained by the gradual loss of the triple-helical structure during gelatinization as a consequence of a decrease of intra- and intermolecular bindings with rising temperatures above the denaturation temperature.[10] The helix-coil conversion leads to the breakage of hydrogen bonds between polypeptide chains, resulting in individual chains or dimers and an increased solubility.[35] Below the denaturation temperature, the triple helical structure of collagen (produced under mild conditions) is almost intact.[35] However, at water temperature of 80°C, the reference film was fully disintegrated within 2 h.

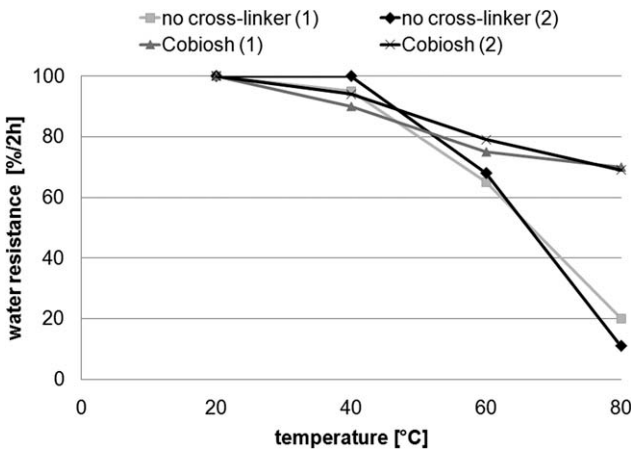

Figure 2 Water resistance of a thermally crosslinked collagen film (Cobiosh) in comparison with the reference collagen film at different temperatures after 2 h, measured gravimetrically (1) and by Sircol™ protocol (2).

The water resistance of the Cobiosh collagen film showed an almost 100% water resistance at 20°C (after 2 h). This result was found to be in good accordance with values reported in the literature by Amin and Ustunol.[5] For instance, Amin and Ustunol[5] reported a solubility of natural collagen casings (Brechteen Co., Chesterfield, MI) of 6.58% after immersing in deionized water containing 0.02% w/v sodium azide as biozid for 24 h at 20°C–23°C (about 0.5% in 2 h). With rising temperatures above 20°C, the resulting data of the Cobiosh film indicated an almost linear correlation between resistance values and temperature. With a resistance of 70% at 80°C after 2 h, the Cobiosh film was much more resistant than the reference film.

Although the mechanism of DHT collagen crosslinking is not completely understood, the improved water resistance of the Cobiosh film is thought to be related to the removal of water from collagen[36] and the formation of amide crosslinks between amine and carboxyl groups. An alternative crosslinking route is through the formation of lysino-alanine following dehydration of serine residues and subsequent reaction of the resultant dehydroalanine with the ε-amino-group of lysine.[37] About 745 of the 3156 residues on the collagen molecule are involved in the reaction, such as residues of aspartic acid, glutamic acid, serine, threonine, arginine, and lysine.[36]

Figure 3 Casted collagen film prepared with collagen suspension A, dried at 37°C.

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis of heat cured collagen showed high-molecular weight aggregates and a decreased solubility in water at 80°C.[38] Determination of the number of free carboxyl and amino groups after DHT treatment showed diminishing values.[36]

After that a collagen film (made from collagen suspension A) crosslinked with a combination of G2, G5, and FA (10% w/w) was prepared and tested as described in the materials and methods section. Figure 3 illustrates such a casted collagen film. The films were stable, pale yellow, transparent and had a thickness of 150 μm.

Primarily responsible for the crosslinking of collagen with aldehydes are the free ε-amino-groups of lysine, beside some others with minor significance.[34] Assuming that a collagen molecule (Type 1) has about 100 ε-amino-groups[39] and that one molecule hardener crosslinks two amino-groups, the theoretically necessary amount of crosslinker is about 0.8% referring to dry matter content. Bowes and Cater[40] used a significantly higher concentration. They crosslinked collagen with formaldehyde (20% w/w), glyoxal (10% w/w), and glutaraldehyde (12% w/w) referring to dry matter content. On the basis of this, an initial crosslinker concentration of 10% w/w referring to dry matter content for this study was chosen.

Figure 4 contrasts the water resistance of the differently crosslinked collagen films (untreated, thermally and chemically crosslinked) after 2-h testing periods at different temperatures measured by Sircol™ Protocol. Compared with the results presented in Figure 2, a chemical crosslinking with the crosslinker combination consisting of G2, G5, and FA (each 10% w/w) and the collagen suspension A produced collagen films, which were almost completely resistant to water even after a 2-h incubation at 80°C, hence showing significantly improved resistance compared with the thermally crosslinked film. The inter- and intramolecular crosslinking with aldehydes shows positive effects regarding water resistance[5,15,20,21] by forming a water-insoluble three-dimensional network via covalent bridges between protein chains.

Kopp et al.[41] observed a decrease in solubility as a function of crosslinking in collagenous material provoked by a decrease in the water binding capacity. A 7% decrease in solubility was reported by Micard et al.,[42] who crosslinked gluten-films with formaldehyde and compared it with unmodified films. Galietta et al.[43] observed the same reduction in solubility for whey protein-based films.

Previous studies have shown that thermal crosslinking such as DHT drying produces weaker bonds, when compared with chemical crosslinking[33,34] although approximately seven times as many residues of the collagen are available for crosslinking using dehydration procedures, when compared with glutaraldehyde crosslinking.[34,36] Abke reported studies, in which the results of thermal crosslinking were compared with the results of chemical crosslinking. Unfortunately, these investigations were difficult to compare among themselves due to the different procedures used (differently prepared basic material, different concentrations, temperatures, and methods of determination to evaluate the degree of crosslinking). Abke reported on the investigations of Weadock et al., who compared the collagenase stability of differently crosslinked (UV, DHT, glutaraldehyde) bovine collagen among each other. Thermal crosslinking led only to slightly improved collagenase stability, whereas glutaraldehyde significantly improved the collagenase stability. Abke also mentioned the studies of Pieper et al., who crosslinked bovine collagen (Type 1) through DHT respective chemical (with) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The DHT treatment did not show any influence on the collagenase stability in contrast to the chemical crosslinking.[34]

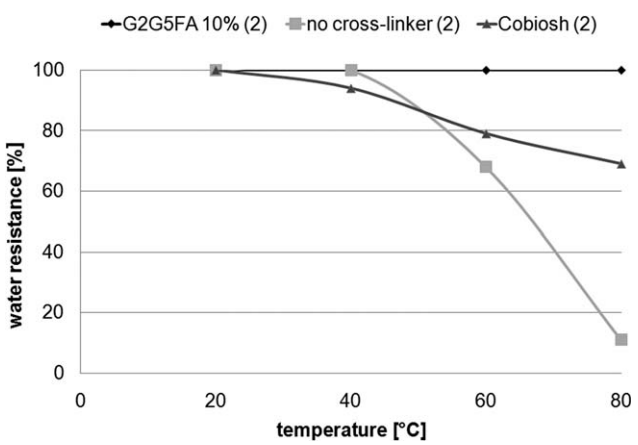

Figure 4 Comparison of water resistance of differently crosslinked collagen films (made from collagen suspension A) after 2-h testing period at different temperatures measured by Sircol™ Protocol (2), FA = formaldehyde, G2 = Glyoxal, G5 = glutaraldehyde, each 10% w/w.

Due to the risk of collagen denaturation, resulting in materials that are more susceptible to solution at the same time, it is difficult to produce more bonds through increasing the exposure time.[33]

As a result, it can be stated and confirmed that it is possible to increase the water resistance of collagen films by crosslinking as previously reported by Courts and Homan[44] or Amin and Ustunol.[5] The increased water resistance is attributed to the formation of additional covalent bonds, as Amin and Ustunol[5] confirmed by SDS-polyacrylamide gel electrophoresis (PAGE). Even Singh et al. [2001] investigated gelatin crosslinking by measuring the solubility of the films. This procedure does not quantify the degree of crosslinking at the molecular level and does not provide information about the mechanism or interactions. Nevertheless, it gives a crude idea about the degree of crosslinking due to the fact that solubility is linearly correlated to the extent of crosslinking.[45] Besides water resistance measurements[46,47] and SDSPAGE,[5] further analytical methods have been reported in previous literature. Courts and Homan[44] verified the crosslinking of gelatin with improved water resistance by measuring the changes in viscosity and optical rotation. However, Carvalho and Grosso[48] determined the number of free amino groups in the modified films, when compared with the native film as indication for the occurrence of polymerization.

Influence of crosslinking agent on water resistance of collagen films

To study the influence of various chemical crosslinking agents on water resistance (see Fig. 1 Step 2), films made from collagen suspension A were prepared as described in the materials and methods section. For the crosslinking procedure, aldehydes such as formaldehyde, glutaraldehyde, and glyoxal, respectively, and combinations of these crosslinkers were used in a concentration of 10% w/w. Therefore, first the amount of crosslinker of 10% w/w was selected following Bowes and Cater,[40] who crosslinked collagen with formaldehyde, glyoxal, and glutaraldehyde with amounts in a range from 10% to 20% w/w. The water resistance was measured at 80°C after 2 h gravimetrically as described above.

Table III summarizes the water resistance (%/2 h) of crosslinked collagen films with different crosslinkers. All crosslinkers, used in a concentration of 10% w/w, resulted in an increased water resistance from 25% (reference film without the addition of a crosslinking agent) to 92% after an 2 h incubation at 80°C water temperature (Table III).

Results show no significant difference between the individual crosslinkers and their combination. However, the reason for the similar water resistances obtained in this study is unclear, and the results were not expected as Bowes and Cater reported a considerably difference in the aldehydes ability to crosslink collagen at optimal pH, concentration and temperature with regard to the number of crosslinks introduced, stability of the crosslink and the bond energy.[36,40] Generally, it is difficult to predict the effectiveness of these crosslinkers as it is influenced by many different factors. Primary responsible for the crosslinking of collagen with aldehydes are the free lysine-ε-amino-groups forming bonds similar to Schiff base, beside some others with minor significance.[34] Marquié et al.,[47] however, reported that formaldehyde for example reacts not lysine specific. Hence, the number of available residues in the collagen molecule plays an important role.[36] Even the reaction rate distinguishes them from each other. Formaldehyde, for example, reacts in two different rapid reaction steps.[47] In addition, the amount of crosslinker is of vital importance. With excessive amounts of glyoxal, it was observed that part of the reagent remains unbound resulting in a decreased cohesion of the polymer matrix and reduced intermolecular forces and thus in a reduced resistance.[47] Weadock et al. reported a production of intramolecular crosslinks in collagen at low concentrations of glutaraldehyde while higher concentrations produced intermolecular crosslinks, due to the formation of long polymeric glutaraldehyde chains. The long chains cannot penetrate into the material. That is why only the surface of the material is crosslinked.[36] Moreover, the pH affects the reaction efficiency. Glyoxal reacts preferentially at alkaline pH,[36] similar to glutaraldehyde, which shows an increasing activity with rising pH from pH 4 to pH 9, with a maximum around ph 8.[47,49] However, Bendino[49] reported an increasing reactivity of formaldehyde with increasing acidity, particularly at pHs of 6.5 and less.

Due to its lower toxicity, glyoxal was selected as crosslinking agent for subsequent studies.

TABLE III
Water Resistance of Crosslinked Collagen Films (Collagen Suspension A) with Different Crosslinkers Measured Gravimetrically After 2 h at 80°C, FA = Formaldehyde, G2 = Glyoxal, G5 = Glutaraldehyde, Each 10% w/w

Crosslinker	Water resistance (%)
Reference	24.8
G2G5FA	94.0
G2FA	93.5
G5FA	92.4
G2G5	93.2
G2	94.0
G5	92.7
FA	92.7

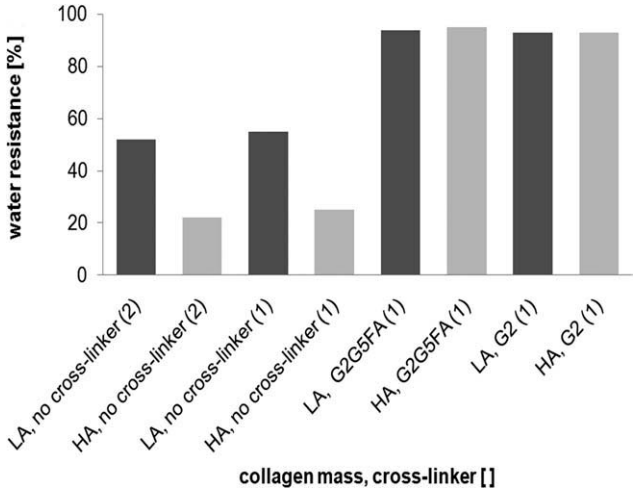

Figure 5 Comparison of water resistance of different collagen suspension (LA = lactic acid, HA = hydrochloric acid) measured gravimetrically (1) and by Sircol™ Protocol (2) after 2 h at 80°C, FA = formaldehyde, G2 = Glyoxal, G5 = glutaraldehyde, each 10% w/w.

Influence of collagen suspension preparation on the water resistance of collagen films

The next step (see Fig. 1 Step 3) examined whether there was a difference in water resistance between differently pretreated collagen suspensions (collagen suspension A acidified with hydrochloric acid and collagen suspension B acidified with lactic acid). Films were prepared from these two masses as described in the materials and methods section, one film each per mass untreated, one crosslinked with a combination of the crosslinking agents G2, G5, and FA (each 10% w/w referring to the collagen dry matter content) and one crosslinked with G2 (10% w/w) after resistance of the crosslinked films was measured gravimetrically. With the samples of the untreated films, both methods of determination were used. Figure 5 enables a direct comparison of the water resistance of untreated or crosslinked collagen suspension A with collagen suspension B. The comparison of a mass treated with hydrochloric acid (collagen suspension A) and a mass prepared with lactic acid (collagen suspension B) produced without the addition of a crosslinking agent, showed a remarkably higher resistance of 55% for the collagen suspension B, compared with collagen suspension A with only $\sim$ 20%–25%. However, the reason for this is unclear. It is suspected resulting from a more severe predamage of the collagen suspension A due to the hydrochloric acid than the lactic acid and the automated manufacturing process (the collagen suspension B is not a standard product and therefore has been produced manually), respectively, a longer interim storage. However, these differences seem to be of no consequence in case of the crosslinked films as no differences concerning the water resistance could be observed. This might be due to the surplus amount of crosslinker (Fig. 5).

Based on these results and the fact that the collagen suspension B possesses a lower corrosion potential, which is relevant when applied in contact with metals, collagen suspension B was chosen for all further experiments.

Influence of crosslinker concentration on the water resistance of collagen films

Next, it was investigated how crosslinker concentrations affect the water resistance of collagen films (see Fig. 1 Step 4). For this purpose, collagen film prepared from collagen suspension B was crosslinked with glyoxal in different concentrations and the water resistance was measured by Sircol™ protocol as described in the materials and methods section.

Figure 6 outlines the water resistance of collagen crosslinked with different glyoxal concentrations after 1 h at 80°C measured by Sircol™ Protocol. With higher crosslinker concentrations, the water resistance, determined by the Sircol™ protocol at different crosslinking degrees, was increased and reached asymptotically 100% at crosslinker concentrations of 10% w/w or more (Fig. 6).

Unfortunately, the findings are difficult to compare with results reported previously in literature, due to the different procedures used (different basic materials, different crosslinker concentrations, temperatures, and methods of determination to evaluate the degree of crosslinking). Nevertheless, attempts will be made to discuss the results. Marquié et al.,[47,50] who examined the lysine content and the percentage of soluble matter of cottonseed flour crosslinked with increasing amounts of glyoxal and formaldehyde, reported a similar dependency on the

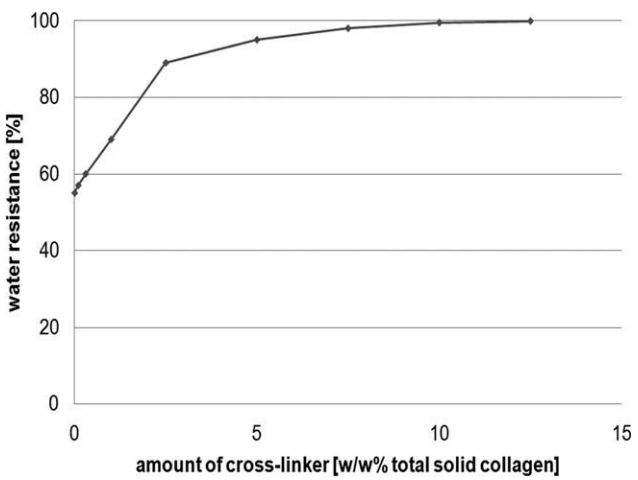

Figure 6 Water resistance of collagen B crosslinked with different glyoxal concentrations after 1 h at 80°C measured by Sircol™ Protocol.

crosslinker concentration, just like Renner [2003], who investigated the crosslinking of gelatin with vinylsulfon (0.1%–10% concerning the dry matter content).[51] With increasing degree of crosslinking, the number of available hydrophilic groups lowers, resulting in a decreased solubility.[52] Another possible reason for the found relations was discussed by Moll et al.,[53] who observed that crosslinking of gelatin molecules is short term in the first stage. Due to this fact, far-off molecules cannot react instantly. He proposed the thesis that higher initial concentrations of the crosslinking agent have improved access to reactive groups. Furthermore, Carvalho and Grosso examined the water resistance of crosslinked gelatin films dependent on the amount of added crosslinker (formaldehyde and glyoxal). They reported decreased water solubility (after 24 h at 25°C) with increasing amount of crosslinker.[46]

Because of the brittleness of the films, the influence of a plasticizer was investigated next.

Influence of plasticizer on water resistance of collagen films

The difficulty caused by the application of a plasticizer to improve plasticity of collagen films is that with increasing levels of plasticizer content, permeability is increased subsequently due to the augmentation of the free volume/intermolecular spacing.[9,19] Furthermore, most plasticizers like glycerine are hygroscopic, hence increase the water content of films.[23] Whether this is also true for lecithin as an example was examined in the next step (see Fig. 1 Step 5). For these investigations, a collagen suspension B was mixed with lecithin (20% w/w), and films were prepared from this mass by crosslinking with G2 (10% w/w) as described in the materials and methods section. Water resistance was determined gravimetrically by Tint, respectively, according to the Sircol™ protocol.

Figure 7 shows a comparison of water resistance curves of a plastified collagen film after a 2-h cooking at different temperatures measured by Sircol™ Protocol and gravimetrically. The determination of the resistance of these films at different temperatures showed a clear downward trend or shift paralleled in both gravimetric determination and the resulting curve determined by Sircol™ Protocol. This shift can be explained by the fact that the plasticizer was released from the film during the cooking process, which leads to a decrease in weight that was not caused by the collagen material as it was previously described for other plasticizers such as gelatin. The detected loss of protein was significantly lower than the loss of weight.[53]

Lecithin is a phospholipid consisting of a backbone of glycerol with two fatty acids and a choline phosphate group, which makes it water-soluble.[54,55] Possibly excess of lecithin, which is not chemically incorporated into the polymer structure can cause the fast release into water. The dissipation of lecithin was not detected by the Sircol™ Protocol (Fig. 7). The analysis of the Sircol™ results implies that this procedure is not suitable to record data as a result of the dissipation of the plasticizer and is therefore limited to the detection of the collagen's water resistance only.

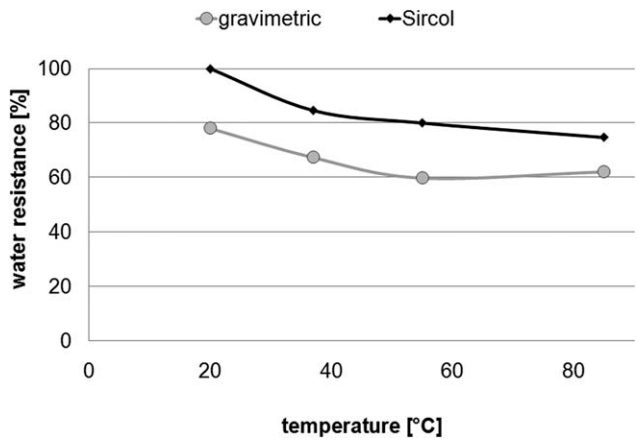

Figure 7 Comparison of water resistance curves of a plastified collagen film (made from collagen suspension B) after a 2-h cooking at different temperatures measured by Sircol™ Protocol and gravimetrically, lecithin (20% w/w), Glyoxal (10% w/w).

In search of elastic films with a high water resistance, other plasticizers need to be investigated. Plant-based plasticizers, such as castor oil or larch resin[56] may possibly come into question here.

General: Comparison of water resistance of differently crosslinked films depending on reaction time

Finally, the chemical resistance of different collagen films depending on their crosslinking type and concentration as well as their reaction time in water at 60°C was examined. To evaluate the film's behavior for an extended testing period, this study was conducted at 60°C deliberately. At a water temperature of 80°C, the reference film would have already been completely disintegrated after 2 h. For this purpose, the thermally crosslinked collagen film Cobiosh, the untreated reference film made from collagen suspension B and collagen films prepared from collagen suspension B crosslinked with 10% w/w G2G5FA, respectively, 10% w/w G2 (for details see material and methods section) were immersed in water for 7 h at 60°C, and the water resistance was measured after 1 to 7 h gravimetrically as described in the materials and methods section.

Figure 8 shows the water resistance as function of the incubation period for differently crosslinked

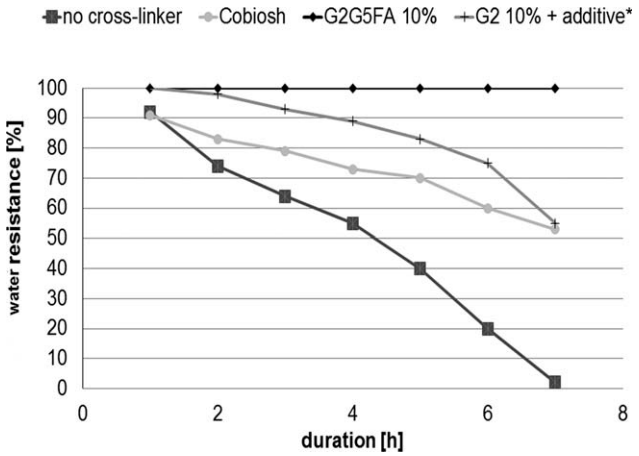

Figure 8 Comparison of water resistance of differently crosslinked collagen films depending on the exposure time at 60°C, measured gravimetrically, FA = formaldehyde, G2 = Glyoxal, G5 = glutaraldehyde (each 10% w/w), additive = lecithin (20% w/w).

collagen films. The untreated collagen film was completely destroyed within 7 h, whereby the resistance declined proportionately to exposure time. The film crosslinked with a G2 solution (10% w/w) and the thermally crosslinked collagen film showed a similar resistance of ~ 55% after 7 h. However, the decrease in resistance of the Cobiosh film proceeded almost linearly with increasing exposure time, whereas the resistance of chemically crosslinked films decreased more rapidly with longer reaction time. The films crosslinked with the crosslinker combination consisting of G2, G5, and FA (10% w/w) stayed stable even after 7 h incubation period at 60°C. As there was no change in resistance observed within the study period, it is assumed that this film may as well resist exposure far longer than 8 h and that the resistance may be substantially higher at lower temperature, respectively.

The results showed once again that it is possible to increase the water resistance of collagen films by crosslinking and that the degree of increase is affected by the type of crosslinking and the amount of crosslinker in case of a chemical crosslinking. Through crosslinking, the formation of covalent bridges between protein chains leads to a water-insoluble three-dimensional network. Depending on the kind of crosslinking procedure and the amount of crosslinker, this network is more or less resistant to solubility.

Further studies are planned to explore the mechanical properties of the films after treatment with water.

Compostability of differently crosslinked collagen films

As compostability can be a decisive criterion for the usage of biomaterials, it was examined to what extent improved water resistance (increased degree of crosslinking) affects the film's biodegradability. Therefore, collagen films from collagen suspension B with different amounts of G2 as crosslinker (0%, 1%, and 10% w/w) were prepared as described in the materials and methods section and tested in accordance with DIN EN ISO 14855-1[29] modified as aforementioned. In the same manner, the compostability of the reference material (cellulose) was examined.

Collagen films produced from collagen suspension B showed rapid disintegration independently of applied crosslinker concentrations. The reference film as well as films with a crosslinker concentration of 1% w/w was already fully disintegrated after 1 day only. The crosslinked films (10% w/w) had significantly shrunk and were severely swollen after 1 day of composting and were completely disintegrated after another day. In this context, the results have shown that increased crosslinker concentrations and improved water resistance do not affect the disintegration of films decisively.

Due to the fact that the experiments described above only prove disintegration, which can be the result of dissolution without degradation, the percentage of biodegradation based on the CO_2 production as definite proof of composting was investigated as described in the materials and methods section in parallel. The results are shown in Figure 9. Independently from the film samples the substances were degraded very rapidly in the first 4 days. This result confirmed the monitored rapid dissolutions of the films. In the following days, the rate of biodegradation decreased significantly. The native, noncrosslinked collagen film showed a percentage of biodegradation of 40% after 38 days at 58°C. According to the DIN-CERTCO list (2004) concerning the degradation properties of biopolymers and their derivates, collagen is aerob and anaerob biodegradable,[57] but it is known as relatively resistant to decomposition on the other hand.[58] The proteolysis

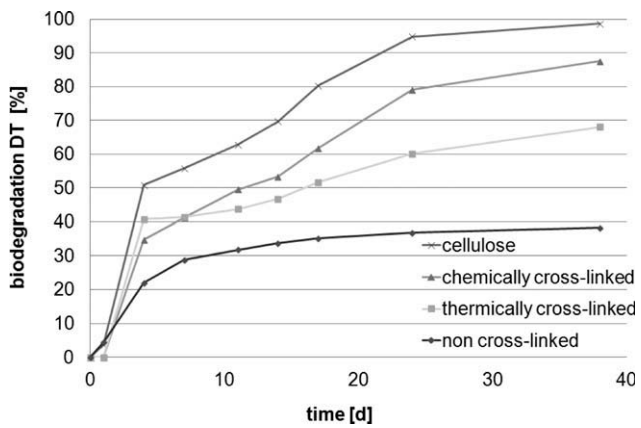

Figure 9 Biodegradability of differently crosslinked collagen films.

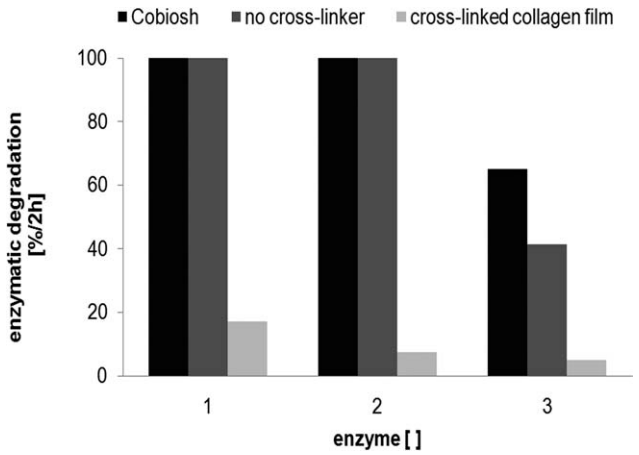

Figure 10 Enzymatic degradation by protease A-01 (1), protease A-02 (2), and trypsin (3) of untreated (no crosslinker), thermally (Cobiosh), and chemically crosslinked (10% w/w Glyoxal) collagen B, measured gravimetrically, protease A-01 and A-08 (500 U/mg or mL, 180 mg/60 mL PBS buffer, pH 10, 40°C), trypsin (2500 USP-U/mg, 185 mg/60 mL PBS buffer, pH 7.5, 25°C).

rate depends beside others on moisture, temperature, and microorganism activity.[58] In soil, the proteolysis of collagen into proteoses, peptones, polypeptides, and amino acids results from bacteria, enzymes, and fungi. Examples for proteolytic bacteria are pseudomonas, bacillus, and micrococcus.[58] Continuing proteolysis leads to low or moderate molecular weight substances and gases such as carbon dioxide. Carter et al.[59] investigated the cadaver decomposition in a controlled setting and found a mass loss of 80% in 28 days at 29°C. In comparison with the relatively moderate biodegradability of the noncrosslinked collagen film, the biodegradability of the thermically crosslinked film was significantly hither (70%). This effect could be explained by the fact that DHT crosslinking entails the risk of partial denaturation/gelatinization of the collagen[5] characterized by a gradual loss of the triple-helical structure[10] and a better biodegradability.[60] The even better biodegradability of the chemically crosslinked collagen film (about 90% after 38 days) is a result of the rapid biodegradability of the lecithin and glycerol in this film.[61,62] The experimental data for the reference material indicated that cellulose is readily biodegradable within 38 days. When interpreting the data, one should keep in mind that estimation of decomposition by determining the CO_2-production ignores C immobilized into the soil microbial biomass and lost as partially degraded intermediates.[63] As such, CO_2-respiration is not a direct measure of degradation.[63]

Enzymatic degradability of collagen films

As enzymatic treatment is another option for degradation of films and therefore a good point for the usage of renewable resources, the optimized films with improved water resistance were then compared with the thermally crosslinked films, and respectively, the reference film. For enzymatic degradation, four different enzymes were selected in this study: First, trypsin, a common digestive enzyme that degrades proteins in the small intestines. Second, pronase,[64] the proteolytic activity of this nonspecific protease is targeted toward native as well as denaturated protein, which typically consists of neutral proteases, chymotrypsin, trypsin, carboxypeptidase, and aminopeptidase. And finally, the two alkaline proteases A-01 and A-08, enzymes derived out of the detergent industry, which are capable of degrading insoluble proteins.[65] Latter mentioned enzymes are particularly interesting in areas of application, where the protection film can be removed simply by washing off the collagen with enzymatic solutions. Initially the enzymatic degradability by protease A-01, A-08, and trypsin of untreated, thermally crosslinked and chemically crosslinked collagen films made from collagen suspension B was tested as described in the materials and methods section. The enzymatic degradability by the different enzymes depending on the crosslinking, measured by Sircol™ Protocol and gravimetrically, is shown in Figures 10 and 11.

The study clearly supports a correlation between the crosslinking and the enzymatic degradability of collagen films. Both, the reference film without the added crosslinker as well as the thermally crosslinked film were degradable by the enzymes protease A-01 and A-08. Once again this is evidence for the weaker bonds of thermally crosslinked collagen

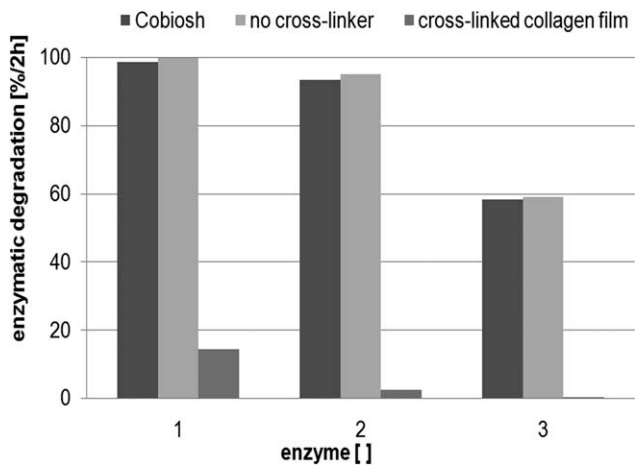

Figure 11 Enzymatic degradation by protease A-01 (1), protease A-02 (2), and trypsin (3) of untreated (no crosslinker), thermally (Cobiosh), and chemically crosslinked (10% w/w Glyoxal) collagen B, measured by Sircol™ Protocol, protease A-01 and A-08 (500 U/mg or mL, 180 mg/60 mL PBS buffer, pH 10, 40°C), trypsin (2500 USP-U/mg, 185 mg/60 mL PBS buffer, pH 7.5, 25°C).

in comparison with chemically crosslinked collagen.[33,34]

Trypsin showed a significantly lower degradation at only 40%–60% in case of the reference film and the thermally crosslinked film. The reduced degradation by the enzyme trypsin can be explained by the fact that collagen of Type I is highly resistant to the proteolytic activity of trypsin.[66] Nevertheless, trypsin was able to slightly degrade the reference film and the thermally crosslinked films. This result can be explained by the fact that possibly the films were slightly denaturated during their production due to the high temperatures used in this process.[33] Trypsin, however, requires denaturation of collagen before it becomes active.[36,66–68]

The crosslinked films, however, showed significantly lower degradation rates by using the enzyme solutions (18% for the protease A-01 and less than 10% for the other two enzymes). The increased stability of the chemically crosslinked collagen film is based on the formation of a stable network by inter- and intramolecular crosslinks.[38,22,67] This crosslinking inhibits the proteolytic enzymes by sterically restricting them from reaction sites. In addition, crosslinks inhibit permeation of the enzyme into the film.[36]

The two different methods (gravimetric determination (Fig. 10) and determination by the Sircol™ Protocol (Fig. 11) resulted in almost identical values.

As degradability of collagen films treated with the enzymes protease A-01, A-08, and trypsin was comparatively low (see Figs. 10 and 11), pronase was used for further testing. It was suggested that pronase, due to its composition of different proteolytic enzymes, would deliver better levels of degradation. Pronase consists of an aminoprotease, which cleaves several amino acid residues from the N-terminus of the collagen, carboxyprotease, which cleaves several amino acids residues from the C-terminus of the collagen and endoproteases like chymotrypsin, which hydrolyses peptide bonds inside the molecule (at least three amino acids residues far away from the terminus).[69] The following figure (Fig. 12) illustrates the correlation between the enzymatic degradation by pronase, depending on the crosslinking degree.

With an increasing degree of crosslinking, adjusted by the amount of added crosslinking agent, enzymatic degradation was clearly decreased. The same effect was observed using both the crosslinker combination as well as an individual crosslinker, however, was more pronounced for the crosslinker combination. Likewise, the treatment of the 10% w/w G2G5FA crosslinked film with pronase did not result in a better degradability. It is suggested that the optimizing procedure to improve water resistance is responsible for this phenomenon. Angele et al.[31] examined the enzymatic degradability of

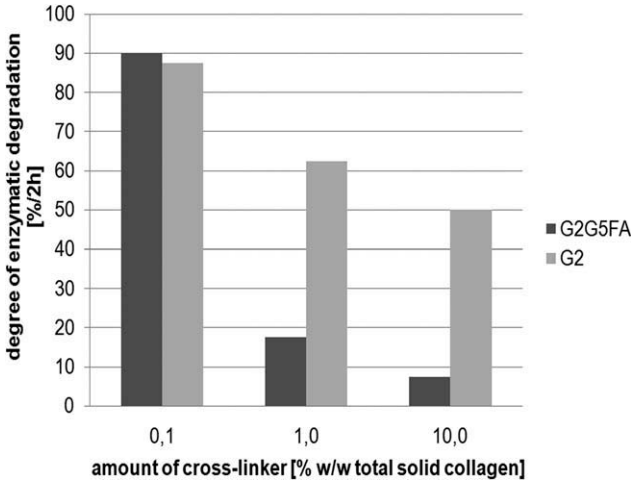

Figure 12 Enzymatic degradation of crosslinked collagen by pronase E from streptomyces griseus (4,000,000 PU/g; 125 mg/60 mL PBS buffer; pH 7.5) within 2 h at 40°C, crosslinked with different amounts of glyoxal, respectively, a combination of formaldehyde (FA), Glyoxal (G2), and glutaraldehyde (G5) (0.1%, 1.0%, and 10% w/w collagen based on dry matter content).

EDC-crosslinked bovine collagen by collagenase. They observed a similar relationship between the enzymatic degradability and the content of crosslinker.

With increasing the degree of crosslinking by increasing the amount of crosslinker, a more and more stable network is formed by the inserted inter- and intramolecular crosslinks.[22,38,67] This leads to a progressive inhibition of the enzyme by sterically restricting them from reaction sites and by reducing the number of specific cleavage sites as well as decreasing penetration of the enzyme into the film.[36,38]

The decreased enzymatic degradability of films with improved water resistance clarify that both factors are mutually exclusive.

CONCLUSION

In this study, the dependency of water resistance of collagen films on different processing factors during crosslinking was investigated. Although a collagen film without crosslinking was almost completely disintegrated after a 2-h cooking period at 80°C (water resistance of 10%–20% after 2 h at 80°C), a thermally crosslinked film (DHT, 1 h, 105°C) showed a significantly higher resistance to water (water resistance of up to 70% after 2 h at 80°C). Chemically crosslinking of collagen with glutaraldehyde, glyoxal, and/or formaldehyde (10% w/w based on the dry matter content), however, led to the highest water resistance (water resistance of up to 100% after 2 h at 80°C). It can be assumed that these films may even resist far longer exposure times. For lower temperatures, these results might even turn out more

obvious, as the correlation between temperature and resistance was demonstrated. With increasing temperature, the water resistance decreases significantly. However, at a concentration level of 10% w/w, no difference between the tested glyoxal, glutaraldehyde, and formaldehyde, respectively, combinations of this crosslinking agents was observed. Significantly increased water resistances of noncrosslinked films prepared from collagen suspensions treated with lactic acid compared with films prepared from hydrochloric acid treated masses were observed. The type of acidifying makes no differences in case of crosslinked collagen films. Glyoxal concentrations below levels of 10% led to a significant decrease of water resistance.

The two selected methods for determining the resistance of the films (gravimetric and Sircol™ Protocol) clearly demonstrated a problem occurring when using a plasticizer such as lecithin in terms of measuring water resistance. The gravimetric determination yielded much lower resistance values than the Sircol™ process in which the dissipation of the plasticizer could not be detected during measuring process.

Finally, it was shown that the improvement of water resistance of the collagen films by chemical crosslinking did affect their degradability by enzyme solutions. The degradability of chemically crosslinked collagen films with lecithin as plasticizer was nearly 90% at 58°C within 38 days.

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References

1. Hamaguchp, P. Y.; Shiku, Y.; Tanaka, M. Pack Sci Technol 2003, 12, 271.
2. Rhim, J. W.; Gennadios, A.; Weller, C. L.; Cezeirat; C.; Hanna, M. A. Ind Crop Prod 1998, 8, 195.
3. Embuscado, M. E.; Huber, K. C. Edible Films and Coatings for Food Applications; Springer: Dordrecht, Heidelberg, London New York, 2009.
4. Han, J. H. Innovations in Food Packaging; Elsevier, Academic Press: San Diego, California, 2005.
5. Amin, S.; Ustunol, Z. Int J Dairy Technol 2007, 60, 149.
6. Yamada, K.; Takahashi, H.; Noguchi, A. J Food Sci Technol 1995, 30, 599.
7. Jones, M. W.; Whitmore, R. A. J Am Leather Chem Assoc 1975, 70, 91.
8. Haroun, A. A. J Appl Polym Sci 2010, 115, 3230.
9. Audic, J.-L.; Chaufer, B. J Appl Polym Sci 2010, 117, 1828.
10. Reich, G. Kollagen, Eine Einführung in Methoden, Ergebnisse und Probleme der Kollagenforschung; Theodor Steinkopff: Dresden, 1966.
11. Tanioka, A.; Jojima, E.; Miyahsaka, K.; Ishikawa, K. J Polym Sci 1973, 11, 1502.
12. Pukhova, Z. I.; Vasil'ev, M. P.; Kotetskii, V. V.; Vol'f, L. A. Fibre Chem 1978, 10, 264.
13. Peña, C.; de la Caba; C.; Eceiza, A.; Ruseckaite, R.; Mondrago, I. Bioresour Technol 2010, 101, 6836.
14. Sothornvit, R.; Olsen C. W., McHugh T. H., Krochta, J. M. J Food Sci 2004, 68, 1985.
15. Kim, S. J.; Ustunol, Z. J Agric Food Chem 2001, 49, 4388.
16. Galietta, G.; Di-Gioia, L.; Guilbert, S.; Cuq, B. J Dairy Sci 1998, 81, 3123.
17. Pol, H.; Dawson, P.; Acton, J.; Ogale, A. J Food Sci 2002, 67, 212.
18. Liu, L.; Kerry, J. F.; Kerry, J. P. Int J Food Sci Technol 2006, 41, 295.
19. Sobral, P. J. A.; Menegalli, F. C.; Hubinger, M. A.; Roques, M. A. Food Hydrocolloids 2001, 15, 423.
20. Gennadios, A.; Ghorpade, V. M.; Weller, C. L.; Hanna, M. A. ASAE Publ 1996, 39, 575.
21. Perez-Gago, M. B.; Krochta, J. M. J Food Sci 2001, 66, 705.
22. Stachel, I.; Schwarzenbolz, U.; Henle, T.; Meyer, M. Biomacromolecules 2010, 11, 698.
23. Orban, J. M.; Wilson, L. B.; Kofroth, J. A.; El-Kurdi, M. S.; Maul, T. M.; Vorp, D. A. J Biomed Mater Res 2004, 68, 76.
24. Maser, F. Oral Information on Aluminiumsulfate; Naturin Viscofan GmbH: Weinheim, Germany, 2011.
25. DIN EN ISO 175, 1999.
26. Biocolor SircolTM Soluble Collagen Assay, Internet manual downloaded from www.biocolor.co.uk, 2010.
27. Lee, D. A.; Assoku, E.; Doyle, V. J Mater Sci: Mater Med 1998, 9, 47.
28. Tint, H. Arch Biochem Biophys 1961, 92, 154.
29. DIN EN ISO 14855–1, 2007.
30. Hoffmann, J.; Řezníčková, I.; Kozáková, J.; Růžička, J.; Alexy, P.; Bakoš, D.; Precnerová, L. Polym Degrad Stab 2003, 79, 511.
31. Angele, P.; Abke, J.; Kujat, R.; Faltermeier, H.; Schumann, D.; Nerlich, M.; Kinner, B.; Englert, C.; Ruszczak, Z.; Mehrl, R.; Mueller, R. Biomaterials 2004, 25, 2831.
32. Junqueira, L. C. U.; Bignolas, G.; Brentani, R. R. Anal Biochem 1979, 94, 96.
33. Lew, D.-H.; Liu, P.; Orgill, D. J Biomed Mater Res 2007, 82B, 51.
34. Abke, J. Dissertation: Verbesserung der Biokompatibilität metallischer Implantate durch kovalente Anbindung einer quervernetzten Kollagenschicht; Naturwissenschaftliche Fakultät—Chemie und Pharmazie, Universität Regensburg, Germany, 2003.
35. Zhang, Z.; Li, G.; Shi, B. J Soc Leather Technol Chem 2006, 90, 23.
36. Weadock, K.; Olson, R. M.; Silver, F. Biomater Med Devices Artif Organs 1983–84, 11, 293.
37. Bello, J.; Riese-Bello, H. Sci Ind Photogr 1985, 29, 361.
38. Gorham, S. D.; Light, N. D.; Willins, M. J.; Bailey, A. J.; Wess, T. J.; Leslie, N. J. Int J Biol Macromol 1992, 14, 129.
39. Shu-Tung, L. Pat. DE69626979T2, Regen Biologics Inc., Menlo Park, CA, USA (2004).
40. Bowes, J. H., Cater, C. W. Biochem Biophys Acta 1968, 168, 341.
41. Kopp, J.; Bonnet, M.; Renou, J. P. Matrix Biol 1989, 9, 443.
42. Micard, V.; Balamri, R.; Morel, M. H.; Guilbert, S. Agric Food Chem 2000, 48, 2948.
43. Galietta, G.; Di-Gioia, L.; Guilbert, S.; Cuq, B. J Dairy Sci 1998, 81, 3123.
44. Courts, A.; Homan, P. J Appl Chem Biotechnol 1972, 22, 1253.
45. Singh, S. et.al. [2001]; Rama Rao, K. V.; Venugopal, K.; Manikandan, R. Pharm Technol 2002, 4, 36.
46. Marquié, C.; Aymard, C.; Cuq, J.-L.; Guilbert, S. J Agric Food Chem 1995, 43, 2762.
47. Marquié, C.; Tessier, A. M.; Aymard, C.; Guilbert, S. J Agric Food Chem 1997, 45, 922.
48. Carvalho, R. A.; de Grosso, C. R. F. Braz J Chem Eng 2005, 23, 45.
49. Bendino, J. H. Champion—An Expanding Encyclopedia of Mortuary Practices, Vol.649; Reseach and Education Department: The Champion Company, Spring field, OH45501, 2003; p 2514.

50. Marquié, C. J Agric Food Chem 2001, 49, 4676.
51. Renner, M. U. Inaugural-Dissertation: Gelatinefilme als Arzneistoffträger; Mathematisch-Naturwissenschaftliche Fakultät, Heinrich-Heine-Universität, Düsseldorf, 2003.
52. Patzsch, K.; Riedel, K.; Pietzsch, M. Chem Ing Tech 2010, 82, 87.
53. Moll, F.; Rosenkranz, H.; Himmelmann, W. J Photogr Sci 1974, 22, 255.
54. Poeggel, G. Kurzlehrbuch Biologie; Thieme Verlag: Stuttgart, 2009.
55. Rassow, J.; Hauser, K.; Netzker, R.; Deutzmann, R. MLP Duale Reihe, Biochemie; Thieme Verlag: Stuttgart, 2008.
56. Felo-Werkzeugfabrik Holland-Letz GmbH, Pat. DE1020080 10755A1 (2009).
57. Amlinger, F.; Fritz, I. Biokunststoffe nachhaltig einführen—erfassen—verwerten; Amt der Niederösterreichischen Landesregierung: St. Pölten, 2008.
58. Dent, B. B.; Forbes, S. L.; Stuart, B. H. Environ Geol 2004, 45, 576.
59. Carter, D. O.; Yellowlees, D.; Tibbett, M. Appl Soil Ecol 2008, 40, 129.
60. Anonymous, Leonardo da Vinci Program: environmentally degradable plastics. Contract Report, Contract No. 1/098/2/05261/PI/II.1.1b/CONT, 1999.
61. Danhong, S., Qiang, H., Wenjum, Z., Liu, X., Bi, S. J Soc Leather Technol Chem 2008, 92, 14.
62. Anonymous, OECD Screening Information Data Sheet SIDS Glycerol, UNEP Publications 1, Paris, 2002.
63. Tibbett, M.; Coarters, D. O.; Haslam, T.; Major, R.; Haslam, R. J Forensic Sci 2004, 49, 1.
64. Roche Applied Science, Pack insert Pronase Cat. No. 10165921001, downloaded from www.roche-applied-science.com, 2011.
65. ASA Spezialenzyme GmbH, Pack insert Protease A-01, EC 3.4.21.62, downloaded from www.asa-enzyme.de, 2011.
66. Gries, G. Z Gesamte Exp Med 1967, 142, 145.
67. Huang, C.; Yannas, V. J Biomed Mater Res 1977, 8, 137.
68. Haurowitz, F.; Tunca, M.; Schwerin, P.; Göksu, V. J Biol Chem 1944, 157, 621.
69. Arnold, U. Habilitationsschrift, Limitierte Proteolyse zur Analyse lokaler und globaler Änderungen der Proteinstruktur am Beispiel von Ribonucleasen; Martin-Luther Universität: Halle-Wittenberg, 2007.