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Soft Collagen-Gelatine Sponges by Convection Drying

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ABSTRACT

This study showed that thermally labile fibrillar collagen could be processed continuously in combination with gelatine as foaming additive by convection drying. The procedure led to stable sponges with similar structural and physical properties as found for freeze-dried collagen samples. The fibrillar collagen remained native, while gelatine acted as foaming additive. The absorbency of the sponges was improved by opening the surface with abrasives. A use as medical device with hemostyptic properties would be possible.

Key words: Collagen, gelatine, convection drying, sponge

INTRODUCTION

Fundamental aspects

Collagen as well as gelatine sponges are broadly used in surgery as wound dressings and hemostyptics (Achneck et al. 2010). The advantage of native fibrillar collagen as hemostyptic compared to many other materials is its very good platelet activation, resulting in a fast aggregation and thrombus formation (Jesty et al. 2008). Furthermore, it is low immunogenic and antigenic in nature (Lynn et al. 2004). In contrast, sponges for hemostasis application manufactured from gelatine induced physical swelling and the hemostyptic properties were lower than that of native collagen (Jesty et al. 2008).

What is the structural difference between collagen suspension, collagen solution and gelatin?

Fibrillar collagen is the main protein part of the skin. Collagen molecules consists of three protein chains that are twisted in a left hand helix to become triple helices. These molecules are stabilized mainly by hydrogen bonds which can be destroyed by heating the molecules above their denaturation temperature (TD). The triple helices form fibrils by staggered side-by-side alignment, leading to the typical collagen cross striation. These fibrils are arranged again in parallel to become longer and thicker fibrils and finally fibre bundles. In hide tissue, these bundles are arranged in 3D networks, whereas in tendon they are ordered in parallel, also in the highest structural degree (Hulmes et al. 2008; Wess 2008). Most of the collagen molecules in tissue are crosslinked at low degree. Chemical nature and site of these natural crosslinks depend on the age and species of the animal as well as the kind of the tissue (Bailey et al. 1998).

Fibrillar collagen of mammalian skin tissue at neutral pH shows a denaturation temperature TD of 55 to 60°C, as measured by differential scanning calorimetry (DSC) (Miles et al. 2005). A soluble native collagen can be obtained from the skin tissue by acidic extraction in the cold. This soluble collagen displays a TD around 40°C (Leikina et al. 2002). For the temperature difference of about 20°C between the TD of fibrillar and the TD of soluble collagen, two main reasons are discussed, which are first, different

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entropic stabilisation of the collagen molecules and, second, different hydration degrees (Kadler et al. 1996).

Heating of soluble collagen molecules above their TD disintegrates the molecules and leads to a protein solution with single protein chains. The molecular weight of denatured soluble collagen can be determined very exactly by size exclusion chromatography (SEC) or SDS-PAGE, leading to defined peaks and bands that correspond to the single protein chains and their di- and trimers, respectively (Meyer and Morgenstern 2003). Collagenous fibrillar tissue shrinks upon heating but it becomes only partly soluble, because the molecules are fixed by their natural crosslinks.

Mincing of collagenous tissue, e. g., unhaired skin in the cold leads to collagen suspensions with a TD at neutral pH similar to that of the collagen tissue. Collagen suspensions are turbid and their average molecular weight MW is very high (several hundred millions Da). Therefore, it cannot be determined by the classical techniques like SEC or SDS-PAGE (Meyer and Morgenstern 2003).

Gelatine is a partial hydrolysate of chemically and thermally treated collagenous tissue. To manufacture gelatine from bovine hide, the raw material is exposed to alkali at pH 12.8 to 14.0 in the cold for several weeks (type B gelatin). This treatment leads to topochemical hydrolysis of the native crosslinks. Porcine skin is treated by mineral acid for one to two weeks (type A gelatin). During this process the main protein chains are cleaved more randomly. Both chemical treatments are followed by extensive washing and hot water extraction during which the triple helices are denatured and main parts of the collagen become soluble. Generally, gelatine is fully soluble. By cooling the warm gelatine solution below a specific setting temperature, it becomes a transparent physical gel, which can be molten again by heating. This setting temperature depends on the degree of structural disintegration during the manufacturing process of the gelatine, but it is always lower than the denaturation temperature of the molecules of soluble collagen. Gelatine solutions with high gel strength usually show setting temperatures between 25 – 30°C. DSC measurements of gelatine gels result in slight peaks with a maximum between 20 and 30°C. This temperature corresponds to the setting temperature, which lays usually 2 to 5°C lower than the maximum of the DSC peaks. The molecular weight of gelatines is broadly distributed but usually centers around 150 kDa or lower (Schrieber and Gareis 2007).

Technical aspects of sponge manufacturing

Collagen sponges are generally manufactured by the lyophilization of aqueous suspensions of minced unsoluble or extracted soluble collagen derived from bovine or porcine skin or from equine tendon. The porosity of freeze-dried sponges depends on the dry matter content of the aqueous suspension to be dried as well as the freezing rate (Friess 1999). However, freeze-drying is expensive and sponges cannot be produced continuously.

Gelatine foams can be prepared by lyophilization as well, but mostly they are obtained by whipping a solution mechanically, followed by cooling the solution to get a gel which is subsequently dried by convection drying (Mansvelt 1979). The latter technique is common in food industry where gelatine is often used as a foam stabilizer to manufacture, e.g., marsh mellows or light airy wine gums. Other techniques to manufacture gelatine foams are, among others, gas bubbling or shaking (Hailing 1981; Campbell and Mougeot 1999). Remarkably, unlike gelatine, native fibrous collagen does not show any foaming ability.

The different physical and thermophysical properties of the various collagen based materials described above may allow the manufacturing of collagen-gelatine foams by conventional drying that combine the foam stabilizing properties of gelatine with the beneficial physiological properties of native collagen. The temperature window between the sol/gel transition temperature of gelatine at around 30° C or lower and the denaturation temperature of native collagen suspension of 55°C and higher may be used to generate stable foams with properties of native collagen.

Therefore, this study aimed at manufacturing collagen-gelatine sponges with a predominant part of native unsoluble collagen combined with collagen based functional additives by applying a continuous convection drying process and thereby circumventing freeze-drying.

MATERIALS AND METHODS

Manufacturing Technology

To find out the best foaming conditions as well as the best foam properties, a couple of different parameters were examined. Mixtures of collagen suspensions, derived from bovine or porcine skin, and gelatine solutions, obtained from the corresponding species, were used as raw material. In these mixtures, the ratio of collagen suspension and gelatine solution was varied. Glycerol was added in varying amounts as a plastiziser. Before foaming, the denaturation temperature of the collagen part of the mixtures was investigated. During the drying process, different values for coating thickness and drying time were applied. Figure 1 gives an overview of the processing technology.

Preparation of the collagen suspensions and gelatine solutions

Collagen suspensions were manufactured from bovine or porcine hides that were obtained from a local abattoir. The hides were split, limed and defatted in a washing drum (Dose, Germany 80 cm). After acidification to pH 2.0 and coarsely grounding by a meat chopper (Nagütec SW 114, Germany), the splits were repeatedly minced with a colloid mill (own construction). The dry matter content of the resulting suspension was ~5%. The treatment led to highly viscous acidic collagen suspensions which could be stored at 4°C for several weeks.

The gelatine solutions were prepared using either type B gelatine (280 bloom, 5,5 mPas) or type A gelatine displaying the same values. Both gelatines were kindly supplied by Gelita Europe. The gelatine (500 g) was swollen in 2500 mL of distilled water at 10°C for two hours and then heated to 40°C until it was completely dissolved. The solutions were prepared at the day of their use.

Foaming, coating and drying

The collagen suspension was diluted with distilled water to a dry matter content of 3,5%, followed by homogenizing at 30°C for 5 min by the use of a rotary stirrer for lab trials. Then the gelatine solution as well as varying amounts of an aqueous glycerol solution (85%) were added depending on the aimed final concentration. The batches were mixed slowly for 30 min at either 30°C (final material with bovine collagen) or 20°C (porcine collagen).

The batches were maintained at 19 to 22°C for the porcine collagen-gelatine mixture and 22 to 30°C for the bovine collagen-gelatine mixture. The batches were foamed physically by mixing with a rotary stirrer (lab trials) or a high speed mixer (Top Mix, Hansa-Mixer, Stuhr Heiligenrode, Germany) that was operated at the highest possible speed. By a doctor blade, this foam was then spread on the surface of an air permeable textile support (polyester) using a Labcoater (Werner Mathis AG, Oberhasli, Switzerland). The coated film was convection dried with a profile of increasing temperature (30-50°C) either in a drying oven (lab trials) or a drying channel (continuous trials), the latter directly arranged behind the coating knife.

Analytical methods

Absorbency

Absorbency was measured by a contact angle measuring system (Krüss DAS 10). An isotonic saline solution (25 µL, 0.9 % in water) was dripped on the foam surface and the time was measured until the drop disappeared.

Differential Scanning Calorimetry

The collagen-gelatine foams were analyzed by Differential Scanning Calorimetry (DSC) to determine the enthalpy of the collagen triple helix and the melting temperature of the gelatine gel as a function of temperature applied to the sample. Samples (4-6 mg) were weighed in a DSC pan and hydrated with an excess of 0.1 M K_2HPO_4/KH_2PO_4 buffer adjusted to pH 7.0. Then the pans were sealed tightly and equilibrated for 5 h. The samples were measured by applying a temperature gradient from 10 to 90°C with a constant heating rate of 5 K/min.

Scanning electron microscopy

Scanning electron microscopy was performed using a Philips XL 30. It was not necessary to coat the samples by sputtering before the measurement.

Bending stiffness

The bending stiffness was measured according to DIN 53864 (Biegesteifigkeitspruefgeraet, Frank Pruefgeraetebau, Germany). A flat sample was clamped into a holder and bent with a defined angle against a force sensor. The force was measured using the following parameters: 30°

bending angle, 3°/s bending velocity, 10 mm bending length and a sample width of 20 mm. The foam side was laid on the outside radius.

Sterilisation

Sterilisation was performed by Gamma Service GmbH (Radeberg, Germany) with the doses of 60 and 100 kGy using a ^{60}Co source.

Wet foam density

Foam density was measured by transferring the foam manually into a weighed glass cylinder with known volume (V), removing the supernatant with a glas rod and weighing again. The mass difference (Δm) was used to calculate the foam density ρ to $\rho = \Delta m / V$ (g/cm³).

Atomic force microscopy

Atomic force microscopy (AFM) was used to analyse the cross striation as marker for the native structure of the collagen part in the collagen-gelatine sponges. The samples were wetted with distilled water and adhered on the microscope slides by drying. To improve the appearence of the collagen fibrils, the samples on the slides were soaked for short time in trypsin solution (0.1% in DSC buffer) in the cold to degrade the gelatine covering the surface. Then the samples were washed with distilled water and dried again. The dry samples were measured directly by the use of an atomic force microscope (Nanowizard II, JPK, Berlin, Germany) using the tapping mode.

RESULTS AND DISCUSSION

The process to prepare the collagen suspension comprised purification and acidification of the raw material (bovine hide or porcine pelt) as well as different grinding and mincing steps. The technology used in this study was adapted from technologies which have been established for the preparation of collagen suspensions to be freeze-dried, e.g., for the manufacture of hemostyptic materials or for convection drying to make collagen foils and casings (Friess 1999; Osburn 2002). The collagen suspension obtained from this process could be used to manufacture collagen-gelatine foams (Fig.1).

In lab trials for foam production, the collagen suspensions were whisked by the use of a rotary stirrer after the addition of glycerol and gelatine solution. The foam density decreased asymptotically with increasing mixing time (Fig. 2). After 15 min a limit was reached and further mixing did not lead to lighter foams. This meant that no more air could be beaten into the foam.

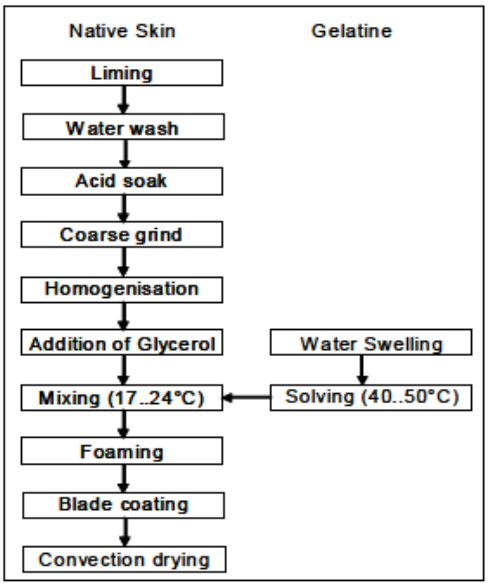


Figure 1 - Manufacturing procedure of collagen - gelatine sponges by convection drying.

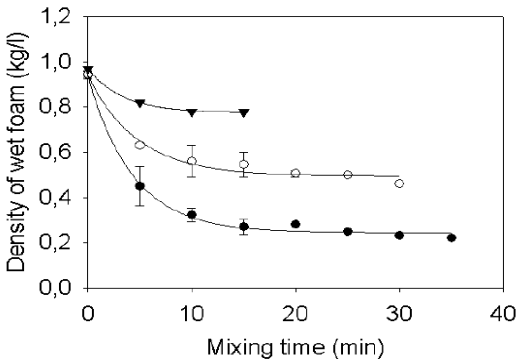


Figure 2 - Wet foam density depends on the mixing time; Ratio Collagen : Gelatine ▼ 1:0,25; ○ 1:0,5; ● 1:1.

However, with higher mixing frequency or variation of the mixing tools, the foam density could be further lowered. Collagen suspension without gelatine as additive showed no foaming capacity (results not shown). Consequently, gelatine was necessary as foaming agent. With increasing gelatine content, foaming was improved and the foams became lighter. The foam density reached 0.2 kg/L when the ratio of collagen to gelatine was 1:1.

The trials for foam production were scaled-up by coating on a continuous working machine. The collagen-glycerol-gelatine mixtures were slowly premixed at the same conditions as mentioned above. Then, the throughput of the mixer was set to 0.5 kg/min. By suitable adjustment of the air-flow, a foam density of 0.14 kg/L was achieved. The range of the bubble sizes for a wet collagen-gelatine foam was between 20 and 500 µm (Fig. 3). This was in the same range as the values of bubble size reported Indrawati and Narsimhan (2008) for a similar continuous working system whipping protein foams from a casein-xanthan gum mixture.

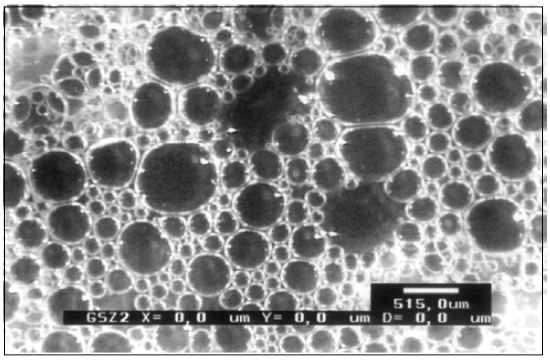


Figure 3 - Bubble sizes of the wet foam.

Neither in lab trials nor during the continuous process the input of mechancial energy was high enough to destroy the foam again. This was described as a problem with labile systems or at very harsh mixing conditions (Hailing 1981), but it was not observed in the collagen-gelatine-glycerol system used in this study. The foam was applied to the substrate by a pilot-scale coating unit. The application was performed at temperatures higher than the gelling temperature of the gelatine to assure that the foam viscosity was low enough. At lower temperatures, gelatine set and the foam sticked at the knife. This led to inhomogenities in the coating layer. Therefore, the knife was heated up to 30°C to prevent this sticking. Directly after the application of the foam, the gelatine of the foam set on top of the substrate. The foam was then dried and the drying temperatures as well as the gradient were adjusted to the humidity content of the foam.

Considering this final drying step during the preparation process, two main questions appeared: (1) Would it be possible to prevent the foam from collapsing during the drying procedure, and (2) would the collagen triple helix of the collagen supension survive the several steps of temperature stress? When collagen suspension alone was convection dried, the capillary forces led to sticking of the collagen fibres and the film appeared compact. However, for the collagen-gelatine mixtures, the foam remained stable when the temperature of the convection drier was adjusted to ambient temperature (20°C). Obviously, setting of the gelatine was achieved at this temperature stabilizing the foam structure. Nevertheless the thickness was reduced from 4 mm (wet foam) to a final thickness of ca. 1 mm for the dry foam.

Fibrillar collagen is thermally labile and denatures between 55 to 60°C. DSC measurements were conducted to check whether the collagen component of the foam still existed in its native form. The results showed that the rehydrated foam consisted of two components. In the DSC thermograms, one very small peak was found between 25 and 35°C and a second peak appeared between 55 and 60°C (Fig. 4 solid line).

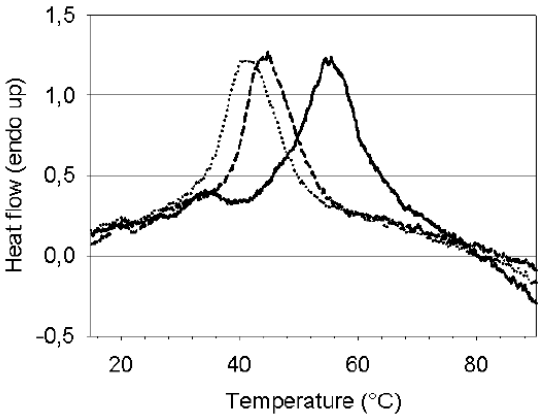


Figure 4 - DSC thermograms of sponge before (____) sterilisation and after application of 60 kGy (- - -) and 100 kGy (•••).

The first peak corresponded to the melting temperature of the gelatine gel. Similar melting temperatures for gelatine gels had been found by others (Michon et al. 1997; Godard et al. 1978) as well as in this study (data not shown). Godard et al. (1978) described that the melting peak of gelatine gels sharpened with increasing setting time. Furthermore, the peak temperature increased when setting time or gelatine concentration were increased. The setting time for the gelatine gel in the foams, that were examined in this study, was

very short compared to usual industrial processes and there was only short time for setting of the gel. Only the period between the coating of the foam and the beginning of the drying process remained for sol-gel transition and setting, which was in the range of seconds. Usually, during industrial processes, the setting time is minutes to hours. However, by increasing the concentration of the gelatine solution up to 20% and by adding glycerol, which is known to shorten the setting time (Oakenfull and Scott 1986), it was possible to achieve sufficient fast setting.

The second peak in the DSC thermograms between 55 and 60°C (Fig. 4 solid line) represented the native collagen component. This temperature range is in good accordance with the temperature that was reported for collagen tissue (Sun and Leung 2008). From the results of the DSC experiments it can be concluded, that the procedure of mixing warm gelatine solution with native collagen dispersion actually led to collagen-gelatine foams with a native collagen component.

The dried foams were further characterized by SEM. Figure 5A shows an image of the cross section of a dry collagen-gelatine foam. The surface appeared to be closed, while the foam showed interconnecting pores and a close connection to the textile substrate. However, when the surface was further examined (Fig 5B), it became obvious that it consisted of an open layer possessing holes that were connected with the inside of the foam. The pore sizes determined by SEM imaging were in the range of 100 µm to 1 mm. This was higher than the pore sizes found for freeze-dried collagen sponges (Fig. 5C) or for gelatine sponges (Hajosch et al. 2010; Barbetta et al. 2010).

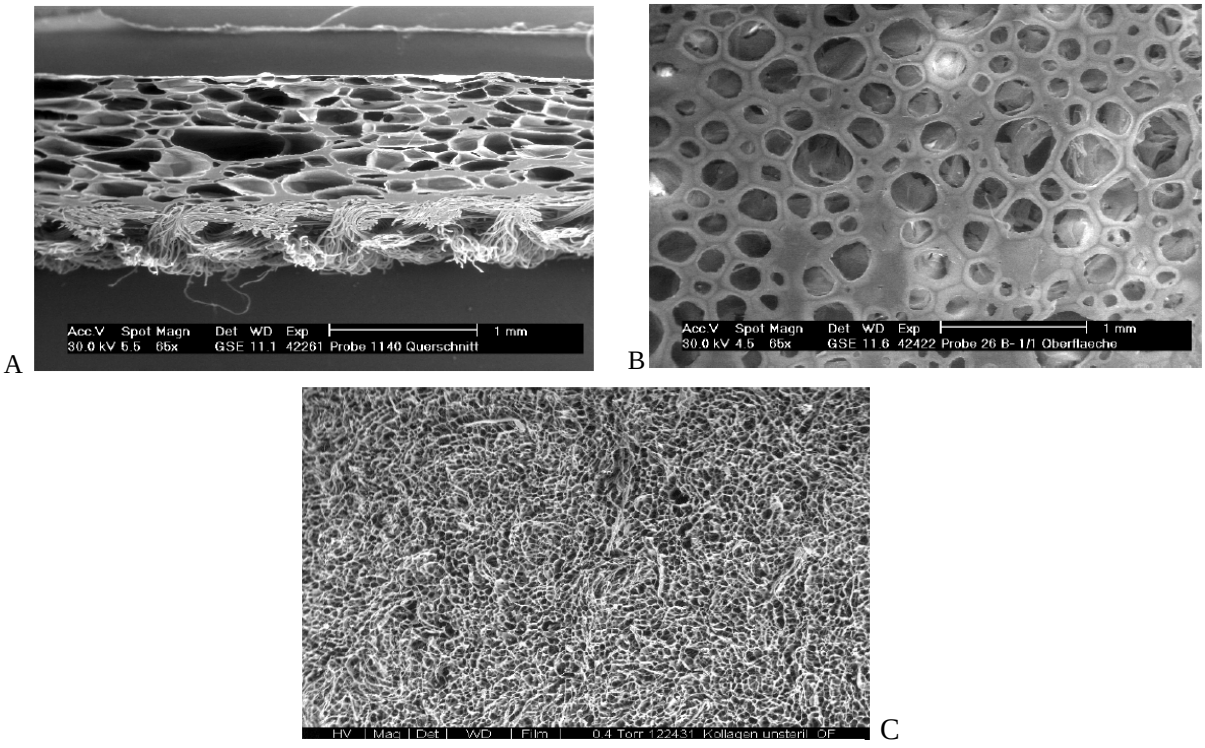


Figure 5 - Crossection (A) and aspect (B) of dry collagen- gelatine sponge; (C) cross section of freeze e dried collagen.

One of the most important criteria of surgical sponges is the absorption time (ta) of aqueous liquids. The sponges have to soak up blood and other physiological fluids during their use as fast as possible. Therefore, ta should be as low as possible. Surprisingly, the open surface as seen by SEM imaging did not allow drops of an isotonic saline solution to be sucked into the sponge (Table 1). However, if the surface was roughened by an abrasive, ta could be shortened. Further improvement was achieved by splitting the surface or by completely removing the textile substrate.

Table 1 - Absorption time of an isotonic saline solution on the surface of collagen-gelatine sponges that were differently treated after drying.

Treatment of Surface	Time [min:s]
None	>10:00
Treatment with abrasive	3:40
Splitting of the surface	1:00
Removement of the textile substrate (measuring backside)	immediately

Beside the surface structure, the hydrophilicity of the collagen component in the foam may influence its absorbing capacity. Therefore, two further parameters, that affect the hydrophilicity of collagen, were investigated regarding their influence on the absorbing capacity of the foam. These were the liming time and an additional oxidative treatment of the raw hide material that was used for the production of the collagen suspension. Increasing the liming time worsened the ta (Fig. 6). During liming, the amide groups (Gln, Asn) are transferred into the corresponding carboxyl groups (Glu, Asp) leading to a decrease of the isoelectric point of the collagen (Bowes and Kenten 1948). Furthermore, long-term alkaline treatment induces the topochemical hydrolysis of some natural crosslinks (Schrieber and Gareis 2007). From this, it might be expected that longer liming times would enhance the absorbing capacity of collagen materials. Other important influences on the collagen by alkaline treatment are not known. Therefore, it remained speculative why the desamidation induced by longer liming times led to an increase of the ta of the collagen-gelatine foams rather than to a decrease.

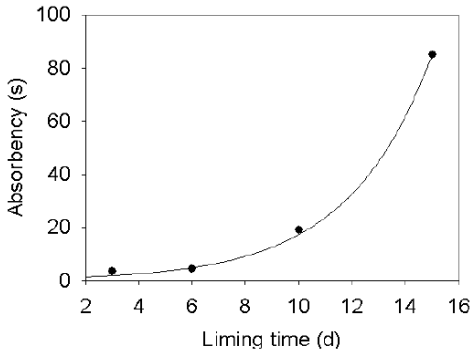


Figure 6 - The absorbency time depends on the liming time of bovine collagen before preparing suspension and subsequently the sponges.

Applying an additional oxidative treatment with hydrogen peroxide before mincing the collagen, improved the ta of the resulting foams (data not shown). It is assumed that the oxidative treatment led to a higher hydrophilicity of the material. However, nothing is known about the exact modifications of the collagen structure or chemistry by hydrogen peroxide. Measuring the amino acid profiles of peroxide treated and untreated collagen did not show any difference other than an expected decrease of the Met content, which could not explain the improved ta. In summary it can be said, that while alkaline treatment of the raw material increased the ta of the collagen-gelatine foams, it was decreased by an oxidative treatment of the collagen raw material. However, the most important parameter that influenced the ta of the collagen-gelatine foams was the surface structure.

The drapability is one further important feature for physicians when using collagen sponges. This drapability corresponds to the softness of the material and can be measured as bending stiffness (Kim and Slaten 1999). The measurements were performed by determining the bending force as a function of the wet foam density. The results showed that with decreasing density of the wet foam, its bending stiffness decreased (Fig.7).

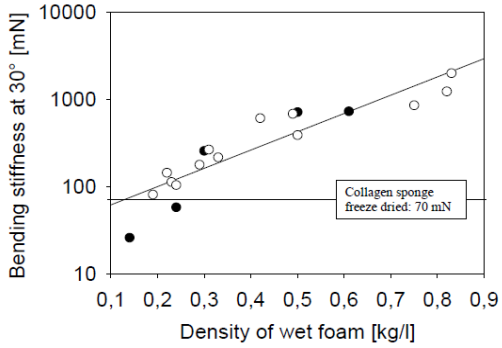
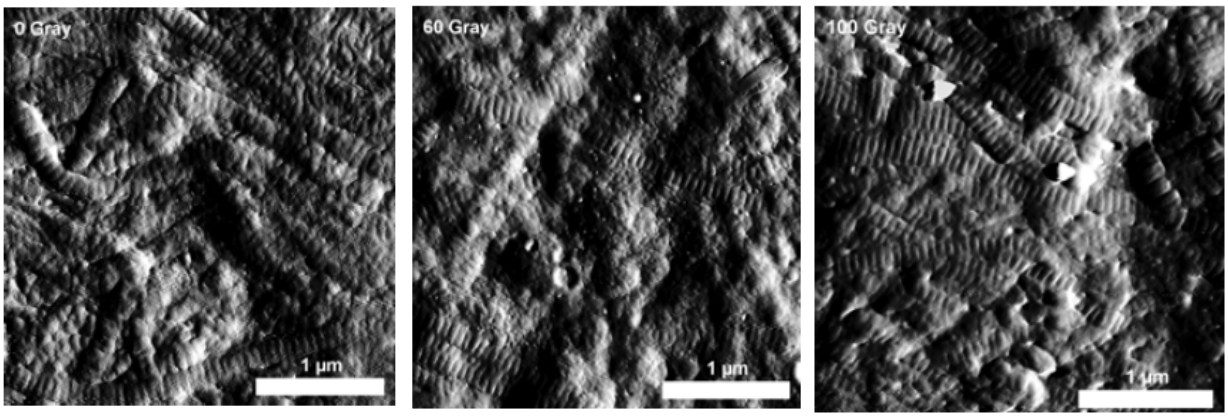


Figure 7 - The bending stiffness of the dry foam decreases with decreasing wet foam density. (◌) bovine collagen / gelatine; (●) porcine collagen / gelatine.

Freeze-dried collagen sponges showed a bending stiffness of 70 mN, which was a very low value compared to that measured for the dried foams that were manufactured by the physical whipping. Nevertheless, a wet foam density lower than 0.3 kg/L led to a bending stiffness being in the same range as measured for the freeze-dried sponges. Thus, it is possible to achieve acceptable softness of the collagen-gelatine foams by applying appropriate preparation parameters.

For medical purposes the foams have to be sterilized before their use. For this, the packed materials are sometimes treated with ethylenoxide, but the most common procedure is the use of γ-ray irradiation (Friess 1999). The effect of sterilisation on the properties of collagen-gelatine foams was analyzed by exposing them to a irradiation with doses of 60 or 100 kGy, which is two to three times higher than the doses usually used for sterilisation. TD of the collagen component in the sterilized foam samples was determined by means of DSC. Depending on the intensity of irradiation, TD decreased from around 58°C for untreated samples to 45°C (60 kGy) and 40°C exposed to 100 kGy (Fig. 4, broken lines). Thus, the native collagen component in the foams was partly destroyed by intensive irradiation. This was already described for collagen tissue samples by Sun and Leung (2008). In the latter study, TD of collagen was directly correlated to the dose of irradiation that was applied. However, the samples of the collagen-gelatine sponges, that were examined in this study, still showed a denaturation temperature higher than that of soluble collagen.
Furthermore, AFM measurements were conducted to analyse the cross striation, a typical key feature of native collagen. This pattern still existed in all the samples that were examined (Figs 8 A-C). No differences could be found between the foams that were not treated and the foams that were treated with γ-ray irradiation. This finding confirmed, that the native fibrous structure of the fibrillar collagen component in the collagen-gelatine foams survived all the different preparation steps.



A B C

Figure 8 - A-C: AFM images of the collagen - gelatine sponges before and after treatment with γ-ray irradiation.

CONCLUSIONS

In this study, foaming of fibrillar collagen suspensions was achieved by the use of gelatine as foaming additive. The foams could be processed continuously into stable collagen-gelatine sponges by coating them to a textile support and applying a convection drying procedure. The sponges displayed similar structural and physical properties than those which were determined for freeze-dried collagen materials. The native fibrillar structure of the collagen component in the foam remained unaffected during the preparation process. To improve the absorption properties, it was most effective to roughen the surface of the sponges either by the use of abrasives or by delamination of the foam from its textile support. Final use properties were excellent with or without the textile support. Thus, the studied collagen-gelatine sponges may be applied as medical devices with hemostyptic properties. Because gelatine as well as collagen preparation are already in use as biomaterials, problems with the approval as a medical device are not expected.

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