Cross-linking by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) of a collagen/elastin membrane meant to be used as a dermal substitute: effects on physical, biochemical and biological features *in vitro*

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Next to *in vitro*-cultured autogeneic keratinocytes for the restoration of epidermis, a suitable dermal matrix is a mandatory component of an artificial skin substitute for the permanent covering of full thickness skin defects. In our model a xenogeneic membrane, consisting of processed native collagen and elastin of porcine origin is meant to serve as a template for the formation of a neo-dermis. In order to improve the resistance of this matrix against enzymatical degradation, we cross-linked it by using the carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) together with N-hydroxysuccinimide. Chemical cross-linking by these agents at two different degrees (shrinkage temperatures 63 °C and 81 °C) had no relevant effect on mechanical features or water-uptake capacity. The time needed for enzymatic digestion was increased by cross-linking. Concerning growth and spreading of fibroblasts and keratinocytes on and within the structure of this membrane, we did not observe a difference between cross-linked and non-cross-linked material (shrinkage temperature 48 °C). We therefore expect that cross-linking by EDC is an effective means to control the degradation of the collagen/elastin membranes *in vivo* without a significant influence on their biocompatibility.

1. Introduction

The replacement of skin, as it becomes necessary, e.g. after deep and extensive burns, remains to present a challenge in plastic surgery. It is undisputed that grafting of autogeneic split thickness skin is still by far the best method to permanently cover extensive skin defects and, therefore, serves as a model for tissue engineering of skin substitutes [1–3]. The introduction of a method for the mass cultivation of keratinocytes by Rheinwald and Green [4] and Green *et al*. [5] provided a magnificent means to re-epithelialize extensive wounds. For years, this method has clinically been used in many burn centers and gained considerable publicity [6, 7]. From frequent reports, e.g. about persistent blister formation or wound contraction, however, it became evident, that cultured epidermal cells, by themselves, are not a sufficient treatment of full thickness skin injuries since this leads to a wound closure of inferior quality [8]. A dermal component is needed to impede these problems brought about by lack of mechanical support or a fragile anchoring of the epidermis to the tissue underneath [9–11].

Numerous types of dermal substitutes have been developed as alternatives to skin autografts, most of which involve a collagenous scaffold of a special architecture which is supposed to provide a three-dimensional message for immigrating connective tissue and vascular cells [12–14]. Often this collagenous matrix is applied in combination with other materials or cells to form a composite graft.

There are basically two different types of collagenous matrices which are used for this purpose. Type I dermal matrices are composed of purified collagen of animal origin which is extracted, homogenized and reconstituted in the form of specially designed three-dimensional structures, e.g. gels or sponges. Often some further additives like chondroitin-6-sulfate are incorporated to increase the strength of the gel or to modulate cellular

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behavior. Special attention is paid to achieve an optimal pore size for cellular immigration. Some models involve the seeding or incorporation of cells, fibroblasts and/or keratinocytes, prior to grafting [15, 16].

When preparing type II dermal substitutes, which are derived from suitable tissue, either from animals or human donors, care is taken to preserve the naturally occurring texture and chemistry of the extracellular matrix as much as possible, i.e. to remove only those components which may cause problems upon grafting due to immunological reasons. The tissues are freed from all disturbing components by gentle cleansing procedures. This can either mean the removal of cells only [17] or other components of the extracellular matrix as well [18, 19]. Since the native structure of this kind of matrix is grossly preserved, type II dermal substitutes exhibit a significantly higher mechanical stability than type I dermal substitutes.

In vivo experiments with both types of dermal substitutes showed, that, with few exceptions, a chemical cross-linking of the basic collagenous material is necessary to control its biodegradation rate and premature resorption into the wound fluids, since this will prevent the reconstruction of dermis-like texture, eventually ending up in scar formation and wound contraction [20–23]. Since the matrix of type I dermal substitutes is inherently fragile, chemical cross-linking adds considerably to their mechanical stabilization [24–27].

Currently, various methods are available for the cross-linking of collagen based materials. Bifunctional agents such as glutaraldehyde or hexamethylenediisocyanate have been widely used for this purpose. However, collagen cross-linked by such substances may release toxic products upon degradation of the oligomeric bridges [28]. The water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) mixed with N-hydroxysuccinimide (NHS), on the other hand, cross-links the collagen matrix by the formation of isopeptides without being incorporated itself. In this way stable covalent linkages are produced directly between activated side-groups of the collagen precluding the release of toxic monomers upon depolymerization [20–27].

We report here on physical and chemical effects of cross-linking by EDC and NHS on a collagen/elastin membrane which belongs to type II dermal substitutes [19, 29]. Since we considered them to be key prognostic parameters for the performance of a dermal substitute *in vivo*, we focused our attention especially on growth and spreading of tissue specific cells.

2. Materials and methods

2.1. Membranes

The basic collagen/elastin membrane as manufactured by Bioplex Medical B.V. (Vaals, The Netherlands) is of porcine origin [19, 22, 29]. It is freed of all non-collagenous and non-elastinous material by a combination of proprietary chemical and mechanical treatments. The membrane consists of approximately 70% collagen and 30% elastin and has a shrinkage temperature of 48 °C. The collagen component of the purified membrane has a nativity of 99.9% as measured by trypsin digestability. The effective removal of proteoglycanes is illustrated by the low level of glucosamine (0.27 mol/1000 mol) and galactosamine (0.11 mol/1000 mol) which are used as markers for heparan sulfate and dermatan sulfate respectively. These were used for the cross-linking procedure, dried and sterilized by ethylene oxide (SRS Laboratories, Coulommiers, France).

The membranes are composed of a mesh of massive bundles of collagen fibers of different length, interspersed with elastin fibers. Concerning swelling and arrangement of the collagen fibers, the membrane texture is rather heterogenous. It has a rough lower aspect which has an open texture which is meant to face the wound and to be colonized, e.g. by fibroblasts and endothelial cells. The other aspect has a smooth surface which is supposed to face outwards to be epithelialized by the application of *in vitro* cultured keratinocytes.

2.2. Cell culture

Skin of patients between age 20–30 years, who underwent surgical breast reductions in the Clinic of Plastic Surgery, Hand and Burn Surgery at University Hospital of the University of Technology, Aachen, was the source of cells, fibroblasts and epithelial cells, for our cell culture experiments in/on the membranes.

Dermal fibroblasts were isolated by explant culture technique from approximately 2 mm^2 pieces of split thickness skin and grown in DMEM (Biochrom KG, Berlin, Germany) supplemented with 10% FCS (v/v) (PAA, Cölbe, Germany).

Epithelial cells were isolated basically according to the method given in Rheinwald and Green [4 and 5] and cultured in DMEM/Ham's F12 (3 + 1) plus 10% FCS (v/v) with the support of lethally irradiated 3T3-cells as feeder-layer. For the biocompatibility studies we used cells of the fifth passage.

Circular pieces of the membranes which had a diameter of 2 cm were rinsed three times in phosphate buffered saline and fixed on the bottom of a specially designed six-well plate (Institute of Pathology, Medical Faculty, University of Technology, Aachen). Each well had an area of 1.77 cm^2. 5×10^5 fibroblasts per cm^2 were seeded onto the rough aspect of the membrane. Six mm punch biopsies were taken after 1, 4, 8, 12, 16 and 20 days and processed for histological and immunohistochemical analysis as well as evaluation by scanning and transmission electron microscope (SEM, TEM).

1×10^5 epithelial cells were seeded per cm^2 on the smooth aspect of the membrane. After a cultivation time of 1, 4, 8 and 12 days punch biopsies were taken and processed and analyzed according to the membranes seeded with fibroblasts.

2.2.1. Histology

Biopsies were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in Paraplast or Technovit® respectively. Thin sections of 5–6 µm were stained either in hematoxyline and eosine (H.E.), Elastica van Gieson (E.v.G.) or Giemsa.

2.2.2. Immunohistochemistry

Biopsies were shock-frozen in a methylbutane-bath in liquid nitrogen. Cryosections were APAAP-stained (Dako) with monoclonal antibodies with different specificities: anti-human collagen type IV, C-1926 (Sigma); anti-human laminin, L-8271 (Sigma), anti-human Ki-67 M 0722 (Dako) and anti-human/pig collagen type VII C-6805 (Sigma).

2.2.3. Transmission electron microscopy (TEM)

Biopsies were cut into strips of widths between 1–2 mm and processed in the following way: (a) fixation overnight at 4 °C in a solution containing 2% each of glutaraldehyde and formaldehyde, respectively; (b) washing in 100 mM phosphate buffer; (c) fixation for 90 min in 3% OsO_4; (d) washing in 100 mM phosphate buffer; (e) stepwise dehydration in increasing concentrations of ethanol (30–100%); (f) incubation for 15 min each in ethanol/propyleneoxide (1 : 1) and pure propyleneoxyde; (g) incubation in propyleneoxide/Epon, overnight; (h) embedding in Epon for 48 h at 70 °C. Ultrathin sections were cut, contrasted with lead-citrate-/uranylacetate and analyzed in a transmission electron microscope (Philipps, TEM 400).

2.2.4. Scanning electron microscopy (SEM)

Biopsies measuring 0.5×0.5 cm were fixed in 4% formaldehyde overnight, washed in 100 mM phosphate buffer, dehydrated stepwise in increasing concentrations of acetone (30–100%) and dried (''critical point''-method). Then the biopsies were sputtered with gold and analyzed in a scanning electron microscope (Philipps, SEM 515).

2.2.5. Determination of the content of free amino acids

By measuring the difference of the content of free amino acids between cross-linked and non-cross-linked materials we determined the degree of cross-linking, either by 2,4,6-trinitrobenzenesulfonic acid analysis or by reaction of free ε-amino-groups with nitrous acid and subsequent amino acid analysis.

Reaction of free ε-amino-groups with nitrous acid: membrane specimens were treated according to the method of van Slyke [30]. One specimen each of the two differently cross-linked membranes (weight 50 mg), were brought into 30 ml of a 30% solution of $NaNO_2$. Under stirring, 10 ml of glacial acetic acid was added. The specimens were incubated in this mixture at room temperature for 24 h under constant stirring. Afterwards they were filtered and washed several times with distilled water. Membranes were dried at room temperature and subjected to complete hydrolysis for subsequent amino acid analysis. By heating of the hydrolysate amino acid groups which had reacted with the nitrous acid do not react any more with ninhydrin. Hence, they cannot photometrically be detected at 440 or 570 nm respectively. The degree of cross-linking was quantified by the difference between the amount of aminogroups of lysine which had reacted with nitrous acid and those which had not.

Reaction with 2,4,6-trinitrobenzenesulphonic acid: three 10 mg specimens of cross-linked and non-cross-linked membrane were each incubated in a mixture of 1 ml 4% $NaHCO_3$ (pH 8.5) and 1 ml of 0.5% (w/v) 2,4,6-trinitrobenzenesulfonic acid for 4 h at 40 °C. Another piece was incubated after adding 3 ml of 6 M HCl to 1 ml 4% $NaHCO_3$. Since in this way the reaction of 2,4,6-trinitrobenzenesulfonic acid with the aminogroups was prevented, this reaction served as control. After incubation the pieces were rinsed in distilled water to remove remnants of unbound stain and incubated in 5 ml each of 6 M HCl for 1 h at 120 °C in the autoclave. After diluting the resulting hydrolysate 1 : 5 with distilled water at room temperature the extinction was measured at 346 nm. Free amingroups were determined on the basis of the molar absorption coefficient of trinitrophenyllysin $1.46 \times 10^4 \text{mol}^{-1} \times \text{cm}^{-1}$ [31].

2.2.6. Measurements of shrinkage temperature

Caused by a cleavage of hydrogen bonds, which stabilize the triple-helical structure, hydrated collagen when heated starts to shrink at a certain temperature or within a small range (2–3 °C) of temperature respectively [32, 33]. Since this shrinkage temperature, T_s, increases if the helical structure is stabilized by covalent bonds it can be used as a measurement for the degree of cross-linking. To determine the shrinkage temperature, strips of the membrane measuring 3×1 cm were tightened in a specially constructed metal device. Upon soaking in water at 20 °C for 5 min the water was heated at a continuous rate of 2 °C per minute until the shrinkage started. Three probes were measured parallel.

2.2.7. Measurement of uptake of water

Due to formation of inter- and intra-molecular bonds the capacity to swell upon uptake of water is influenced by cross-linking. To measure this capacity a piece of membrane weighing 1 g was soaked in water for 30 min at room temperature. Afterwards excess water was removed by rolling the probe out between two pieces of filter paper. After weighing these probes they were dried in an incubator at 100 °C and weighed again until reaching a constant value. Capacity was determined by subtracting the weight of the dried material from that of the swollen. This experiment was repeated three times with five parallel probes each.

2.2.8. Measurement of tensile strength and elongation at break

Tests for tensile strength and elongation at break were performed with (a) dry membranes, (b) membranes incubated in PBS, (c) membranes after incubation with cells, and (d) human split thickness skin (0.3 mm thick remnants from elective surgery for breast reduction). Test samples were cut to strips of 2.3×0.5 cm. The initial

cross-sectional area was measured by a micro-precision device. At least 10 samples were measured under normalized climatic conditions (23°C ± 5%, relative Humidity 50% ± 5%). Test samples were fixed in a JJ Loyd T5K-mechanical tester and a crosshead speed of 13 cm min^{-1} was applied until rupture of the test specimens occurred. In this way the strength F and the elongation Δ1 were plotted by an x-y-plotter 7507 (Hewlett Packard) for each specimen individually.

2.2.9. Statistical evaluation

For statistical evaluation we applied the Kruskal-Vallis test and/or the Wilcoxon-two-sample test.

2.2.10. Measurement of enzymatic degradation

In order to determine the change of resistance against proteolytic enzymes after cross-linking, the membrane was exposed to collagenase from Clostridium histolyticum (Sigma, Deisenhofen, Germany). Enzymatic digestion was determined by photometrically measuring the amount of free hydroxylysine and by weighing the non-degraded membrane [23]. Experiments were repeated three times with three probes each and three controls. A piece of membrane weighing 10 mg was incubated in a closed vial in 0.5 ml of 0.1 M Tris-HCl, 5 mM $CaCl_2$ and 0.05 mg/ml NaN_3, ph 7.4 at 37 °C for 10 min. 0.5 ml of a solution of collagenase (100 U/ml) was added. 0.5 ml Tris-HCl without enzyme was added to the controls. Vials were incubated at 37 °C until the reaction was terminated after 0.5 h, 2.5 h, 5 h, 10 h and 25 h by addition of EDTA. Vials were centrifuged at 600 × g for 10 min. 0.1 ml of the supernatant was mixed with 0.9 ml of 6 N HCl. Hydrolysis was accomplished by incubation at 110 °C for 48 h. The hydroxyproline content of the supernatant was determined.

The non-degraded residue of the membrane was rinsed repeatedly in distilled water, dried and weighed.

3. Results

Cross-linking was observed physically by increasing shrinkage temperature of the material. The selected conditions resulted in cross-linked membranes with a shrinkage temperature of $T_s = 63$ °C and $T_s = 81$ °C, whereas the non-cross-linked membrane had a shrinkage temperature of $T_s = 48$ °C (Fig. 1).

The degree of cross-linking was chemically determined by qualification of the free and cross-linked amount of ε-amino-groups of lysine. Quantification was either performed by 2,4,6-trinitrobenzenesulfonic acid analysis as well as by reaction of free ε-amino-groups with nitrous acid and subsequent amino acid analysis. According to these measurements the amount of free amino groups decreased with increasing shrinkage temperature. The results of the 2,4,6-trinitrobenzenesulfonic acid analysis were representative: for the membrane with the shrinkage temperature of $T_s = 63$ °C a relative degree of cross-linking of 32% and for the membrane with the shrinkage temperature of

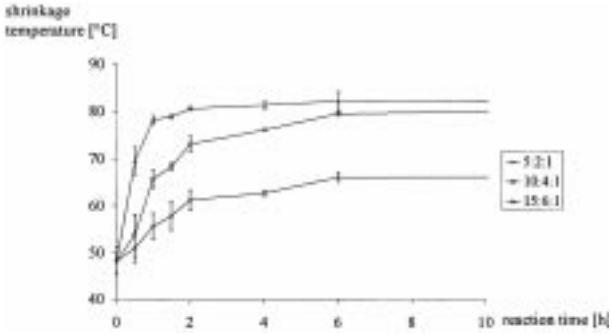

Figure 1 Shrinkage temperature of collagen-elastin-membranes (CEM) as a function of reaction time during cross-linking of non-cross-linked CEM with three different concentration ratios of the carbodiimide 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide to the number of carboxy groups of aspartic and glutamic acid of the CEM (EDC:NHS:COOH).

$T_s = 81$ °C a relative degree of cross-linking of 70% of the ε-amino-groups of lysine was determined (Fig. 2).

Effects of cross-linking were measured on the thickness and ability of the membranes to take up water. The differences did not reach significant levels (Fig. 3).

With regard to the high mechanical requirements which human skin has to meet we examined the mechanical properties of the non-cross-linked and the cross-linked membranes in terms of tensile strength σ and elongation at break ε in dry form, after incubation in PBS and after 20 days of *in vitro* cultivation with fibroblasts and compared them with human split thickness skin. The moduli of extension were determined for the non-cross-linked dry membrane.

In dry form the material showed an increase in tensile strength with increasing degree of cross-linking/ $T_s = 48$ °C: $\sigma = 8.79$ N/mm^2, $T_s = 63$ °C: $\sigma = 11.87$ N/mm^2, $T_s = 81$ °C: $\sigma = 8.79$ N/mm^2) (Fig. 4). After incubation of the membranes in PBS the tensile strength of non-cross-linked samples increased up to more than 100% whereas the tensile strength of both cross-linked samples decreased by about 40% (Fig. 5).

In comparison to the control, cultivation of fibroblasts on non-cross-linked membranes *in vitro* caused a

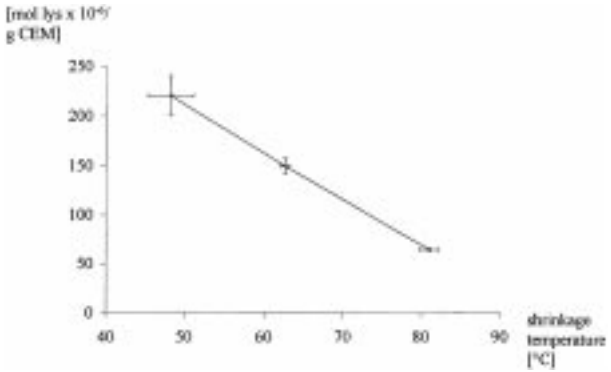

Figure 2 Content of lysine of non-cross-linked and cross-linked CEM ($T_s = 63$ °C and $T_s = 81$ °C) as determined by photometric measurement of trinitrophenyl derivative, the reaction product of ε-amino-groups with 2,4,6-trinitrobenzenesulfonic acid.

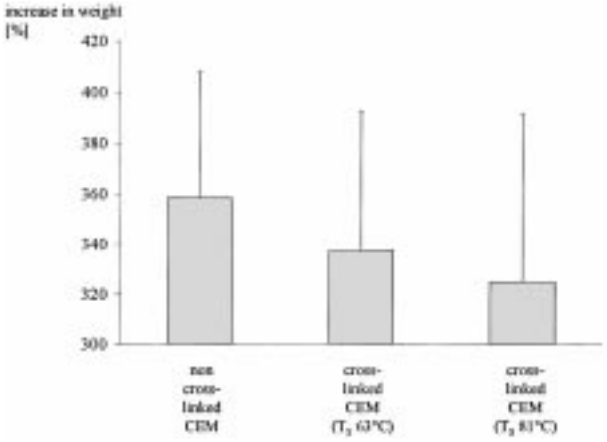

Figure 3 The figure shows the increase in weight of non-cross-linked and cross-linked (shrinkage temperatures $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$) CEM by uptake of water.

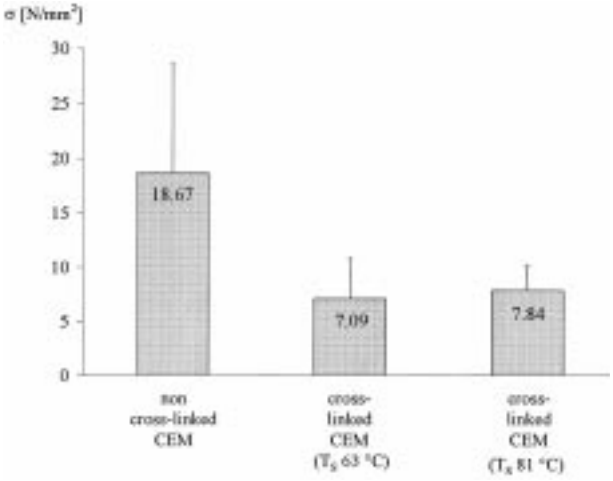

Figure 5 Tensile strength of PBS-incubated non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$.

massive decrease of tensile strength of more than 80%. This is likely due to the enzymatic degradation of the biochemical less stable non-cross-linked membrane (Fig. 6).

As expected, with increasing degree of cross-linking, producing an increased strutting of the matrix of dry samples, a decrease of the average elongation at break was registered (Fig. 7). Membranes incubated in PBS obtained a significant higher elongation at break than dry membranes. In comparison to membranes incubated in PBS, cultivation of membranes with fibroblasts resulted in a significant higher elongation at break only in non-cross-linked samples and cross-linked samples with $T_s = 63\,^{\circ}\mathrm{C}$. This implies that the higher the degree of cross-linking, the lower the effect of *in vitro* cell cultivation on the elongation at break of the membranes (Figs 8 and 9).

The results of tensile strength and elongation at break of human split thickness skin ($\sigma = 5.41\,\mathrm{N/mm^2}$, $\varepsilon = 48.19\%$) and membranes are partly similar to each other. In dry form tensile strength of membranes was significantly better than the tensile strength of human split thickness skin. Although tensile strength of cross-linked samples decreased after incubation in PBS it did not decrease below the values of human split thickness skin. We also measured the enzymatic degradation of the material with collagenase gravimetrically by the loss of weight of the samples (Fig. 10) and photometrically by determination of the hydroxyproline content of degraded collagen (Fig. 11). The collagen of non-cross-linked samples was almost completely degraded within a short time period, while about 90% of the original existing collagen of both cross-linked membranes remained undegraded, indicating the increased biochemical stability caused by cross-linking.

To study the influence of cross-linked in comparison to non-cross-linked membranes on cell vitality and proliferation, membranes were inoculated *in vitro* with human dermal fibroblasts (Fig. 12a–c) and epidermal cells (Fig. 13a–c). During the time of cultivation on either side of the membrane (the smooth surface seeded with epidermal cells, the rough surface seeded with fibroblasts) an increase in the number of cells could be detected, which was accompanied with an increase of

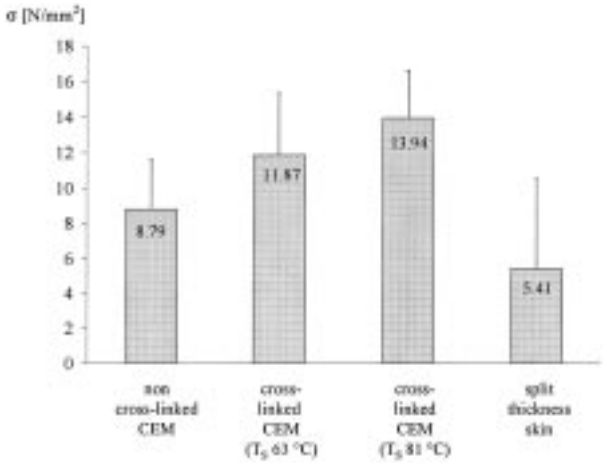

Figure 4 Tensile strength of dry non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$ in comparison to human split thickness skin.

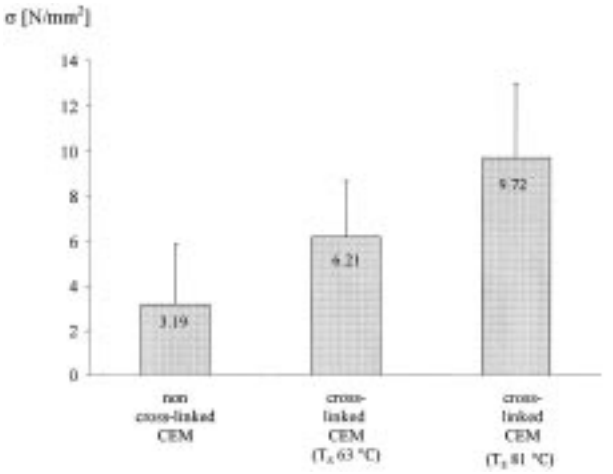

Figure 6 Tensile strength of non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$ which were incubated with fibroblasts *in vitro* for 20 days.

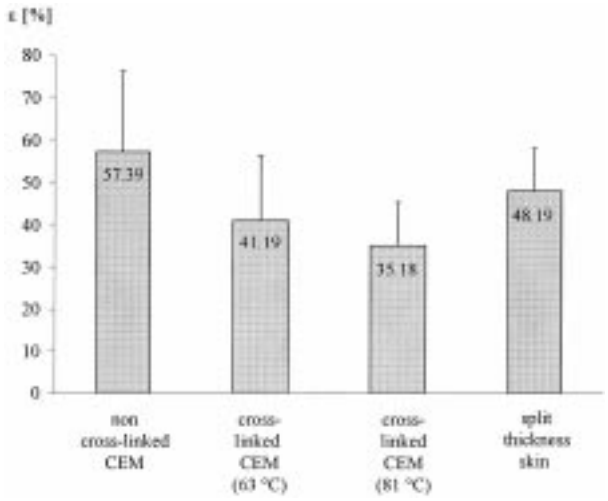

Figure 7 Elongation at break of dry non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s >= 63\,^{\circ}C$ and $T_s = 81\,^{\circ}C$ in comparison to human splitthickness skin.

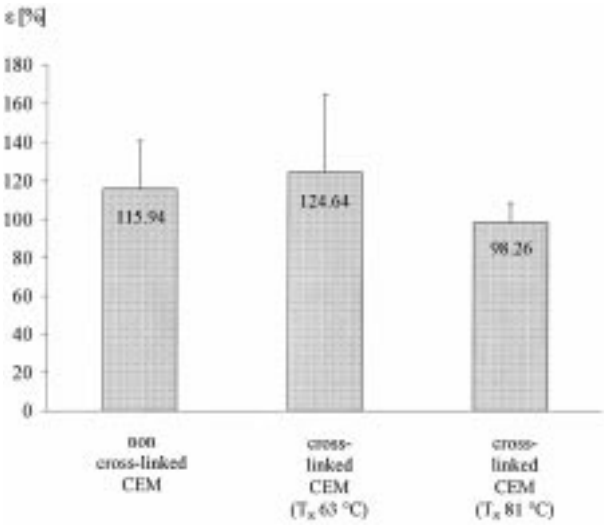

Figure 9 Elongation at break of non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}C$ and $T_s = 81\,^{\circ}C$ which were incubated with fibroblasts *in vitro* for 20 days.

total vitality of cells. Finally, in the case of the epidermal cells, multiple cell layers were formed. Moreover, the migration of fibroblasts resulted in a colonization of the membrane in its complete thickness. We did not observe the production of a basal membrane, however, probably due to the restricted culture conditions. Other components of the extracellular matrix, on the other hand, were produced by the fibroblasts on, as well as within, the texture of the membrane. Condition of the cells, as could be judged especially by TEM (Figs 14 and 15) and SEM (Figs 16 and 17), appeared to be rather heterogeneous. Nevertheless, the mostly well developed cells, having normal cell organelles indicated the conditions to be physiological. We did not find any significant difference between the behavior of epidermal cells and fibroblasts on cross-linked or non-cross-linked membranes.

4. Discussion

As early as 1942 Padgett, after a life-time of experience in plastic surgery, stated that ''With the exception of certain anatomic factors, the base on which the skin lies tends to contract in direct proportion to the thinness of the graft which is applied'' [34]. About 10 years later Billingham and Reynolds, having done a series of experiments concerned with the epithelialization of skin defects, came to the conclusion that ''within a certain range, wound contraction is directly connected to the thickness of the grafted dermis or dermal substitute as well as its resistance against enzymatic degradation'' [10]. These two statements reflect that the importance of a suitable dermal component for the reconstruction of a skin has since long been recognized in plastic surgery. The grafting of epithelial cells alone, either in the form of coherent sheets, as single cell suspensions or grafted on

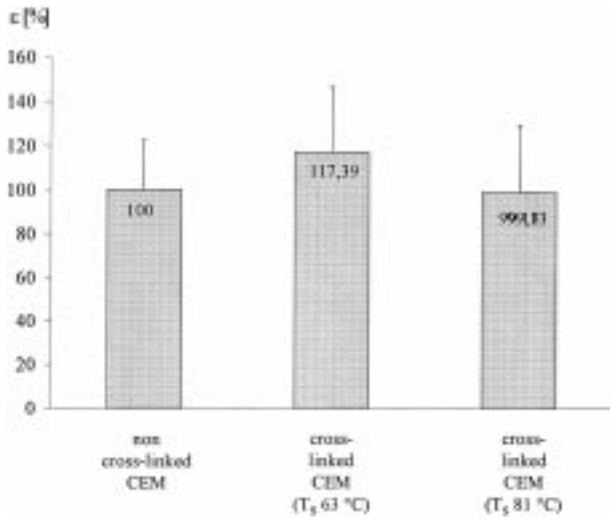

Figure 8 Elongation at break of PBS-incubated non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}C$ and $T_s = 81\,^{\circ}C$ in comparison to split thickness skin.

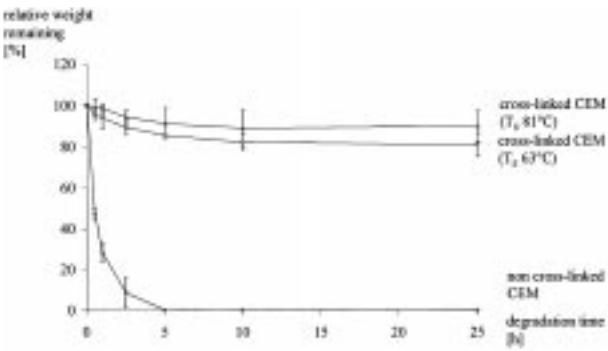

Figure 10 Degradation of non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}C$ and $T_s = 81\,^{\circ}C$ by collagenase (concentration of collagenase = 5 Units pro mg CEM as a function of time. The degradation is monitored by determination of the relative weight of remaining CEM.

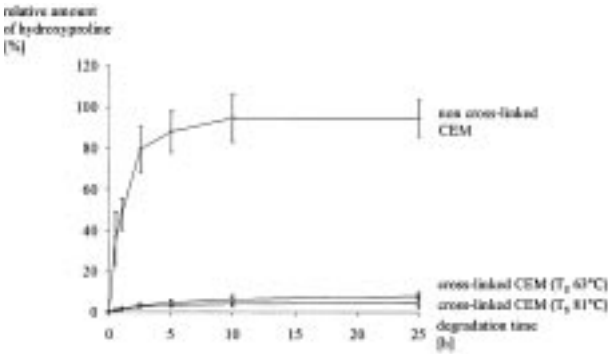

Figure 11 Degradation of non-cross-linked and cross-linked (shrinkage temperatures $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$) CEM by collagenase from Clostridium histolyticum (concentration of collagenase = 5 Units/mg CEM as a function of time. The degradation is monitored by determination of the relative amount of hydroxyproline of the degraded and hydrolyzed CEM.

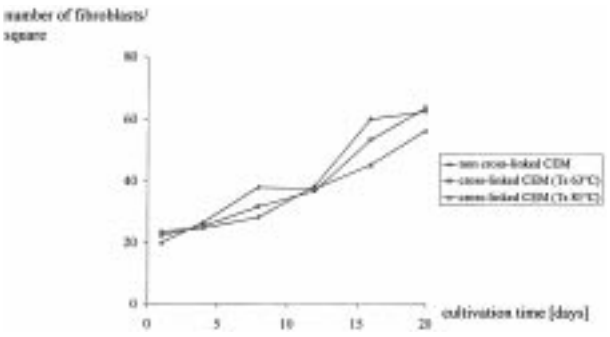

Figure 12a Mean number of human dermal fibroblasts cultivated on non-cross-linked CEM and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$ as a function of cultivation time. One square ist defined as the area of a histological cross-section, which is found beneath a surface length of the embedded CEM of 0.5 mm.

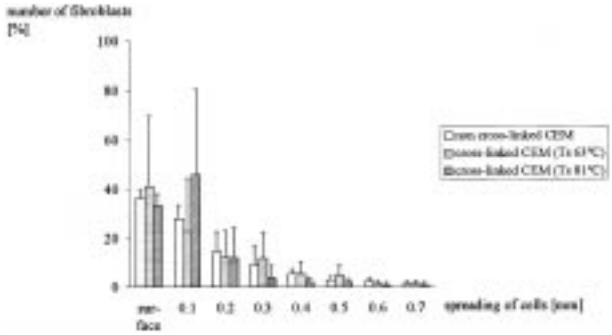

Figure 12b Spreading of human dermal fibroblasts after 20 days of cultivation in non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$. In order to compare the spreading of the cells on non-cross-linked and cross-linked CEM, the total number of cells was normalized to 100%.

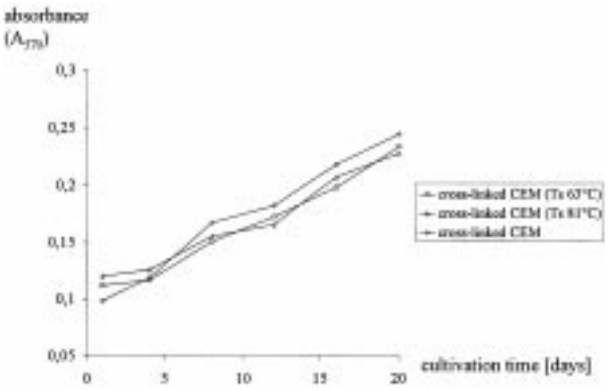

Figure 12c Average vitality of human dermis fibroblasts, cultivated *in vitro* on non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$, as measured by the absorption of reduced (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromid.

special carrier-foils, solve only half the clinical problem since they contribute nothing to the reformation of the missing dermis. An ideal graft would contain mesenchymal elements whose proliferation and fiber-forming activities would produce an adequate substitute for the dermis. Though this was a conclusion of an experimental work and of clinical experience half a century ago, it did not lose a bit of its actuality.

These requirements are best met by the dermal component of grafted autogeneic skin, however, in the case of extensive wounds there is a need to utilize alternative matrices. Collagen is the basic material for the majority of dermal substitutes today. No matter whether this collagen has been extracted from animal tissue, cleansed and reconstructed under controled conditions or whether the native texture has been left intact as much as possible, in all models emphasis is always put on the transmission of a three-dimensional message from the matrix to the colonizing cells. Currently, the characterization of this message on a molecular level, i.e. a key problem in tissue engineering, is in an early phase [35–37]. It seems to be already obvious, however, that a successful transmission of this message requires the matrix to exhibit a certain minimum mechanical and chemical stability.

Chemical cross-linking is an often applied strategy to increase the resistance of purely collagenous biomaterials against the high levels of proteolytic activities which occur especially during the initial phases of wound healing. Since they are known to be very effective, bifunctional reagents like glutaraldehyde have been widely applied for cross-linking of biomaterials in the past, however, a negative effect on their biocompatibility can hardly be avoided [28]. More recently promising results have been described upon cross-linking by carbodiimides like EDC [20–27]. It has been reported that by using EDC in combination with NHS it is possible to achieve degrees of cross-linking comparable to glutaraldehyde [23, 25, 38].

Based on our experiences with EDC cross-linking collagenous cell-carrier foils (data not shown), it was the purpose of this study to evaluate the effects of this method on a new kind of collagen/elastin membrane which, in its non-cross-linked form, had shown promising results in previous experiments. On one hand it was our goal to significantly increase the resistance of this matrix against proteolytic enzymes in order to prevent premature degradation, on the other hand we did not want to change the biocompatibility and those favorable features of the membrane relevant for its clinical application.

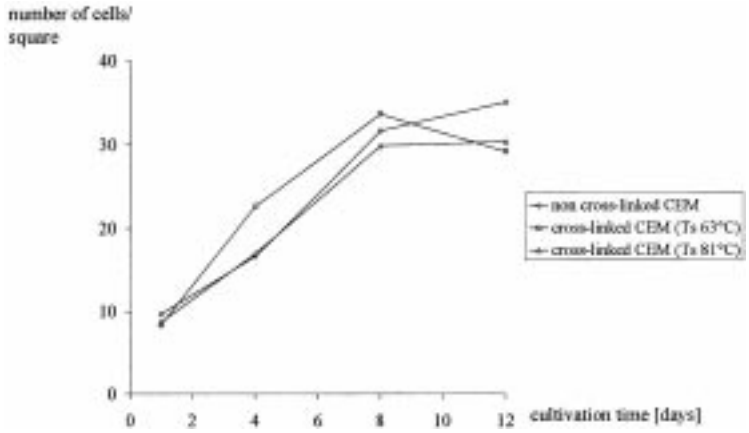

Figure 13a Mean number of human skin epidermal cells cultivated on non-cross-linked CEM and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$ as a function of cultivation time. One square ist defined as the area of a histological cross-section, which is found beneath a surface length of the embedded CEM of 0.5 mm.

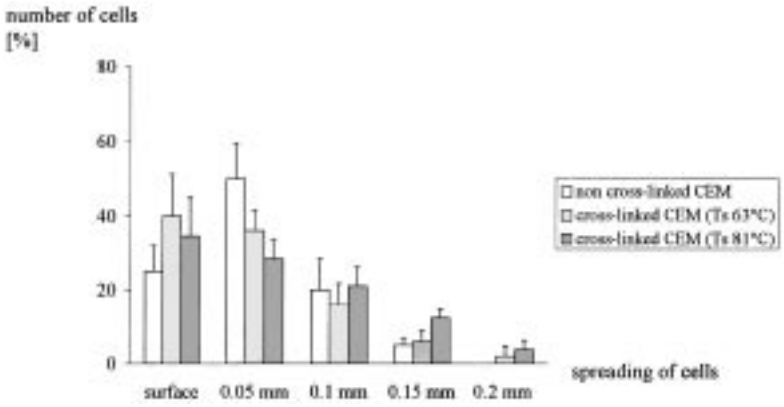

Figure 13b Spreading of human skin epidermal cells after 12 days of cultivation in non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 83\,^{\circ}\mathrm{C}$. In order to compare spreading of the cells on non-cross-linked and cross-linked CEM, the total number of cells was normalized to 100%.

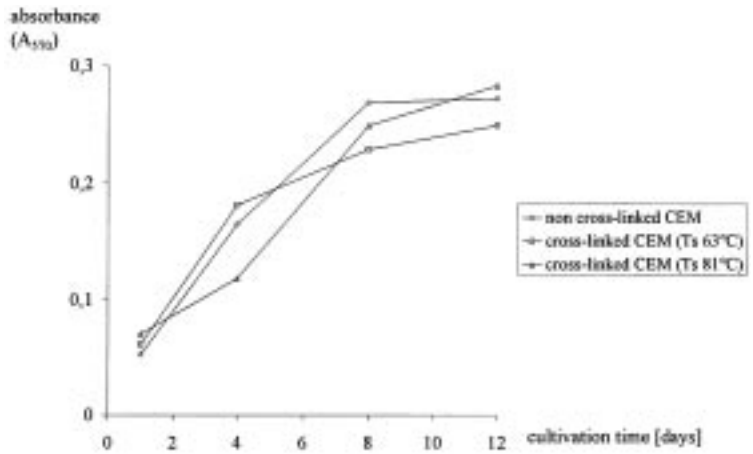

Figure 13c Average vitality of human epidermal cells, cultivated *in vitro* on non-cross-linked and cross-linked CEM with shrinkage temperatures of $T_s = 63\,^{\circ}\mathrm{C}$ and $T_s = 81\,^{\circ}\mathrm{C}$, as determined by measuring the absorption of reduced 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromid.

As shown by the increase of shrinkage temperature and by determination of free amino acids we achieved different degrees of cross-linking. Consequently, this resulted in a considerable increase of chemical stability, as measured by degradation of the material by collagenase from Clostridium histolyticum. In this context it is interesting to mention the massive decrease of tensile strength of the non-cross-linked membrane upon cultivation with fibroblasts, which we would like to interpret as an indication for the enzymatical degradation by the cells.

Since the native membranes which we used in these experiments have mechanical properties comparable to split thickness skin, there was no need to provide further mechanical stability, as would have been the case for reconstituted collagenous materials of type I dermal substitutes. Accordingly, characterization of mechanical features such as tensile strength and elongation at break,

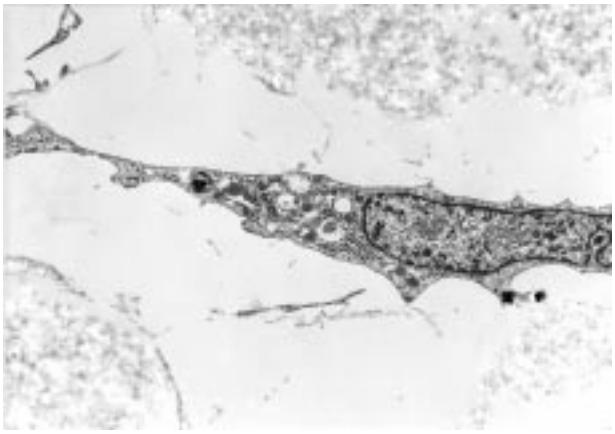

Figure 14 TEM micrograph of human skin fibroblast inbetween the matrix, cultivated on cross-linked CEM (shrinkage temperature $T_s = 63°C$) for 20 days *in vitro*, in a depth of approximately 30 µ of the rough aspect of the membrane. (Original magnification × 5000.)

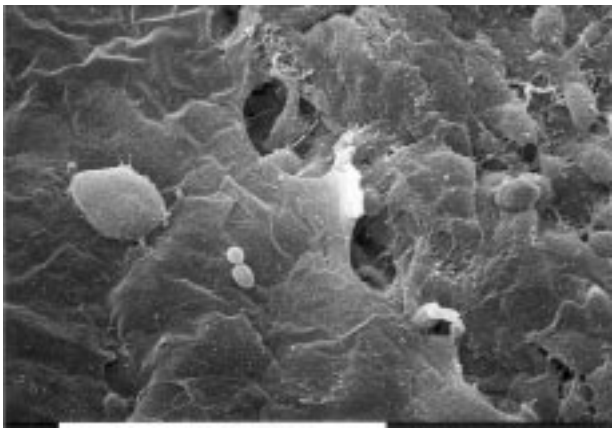

Figure 17 REM micrograph of the smooth aspect of a cross-linked CEM, covered by a confluent layer of human epidermal cells after cultivation for 12 days *in vitro*, (White bar = 0.1 mm.)

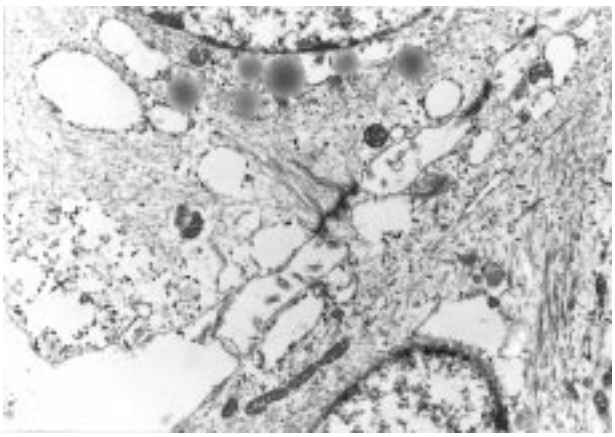

Figure 15 TEM micrograph of human epidermal cells, cultivated on the smooth aspect of the CEM (shrinkage temperature $T_s = 63\,°C$) for 12 days *in vitro*. Cytoplasm of the cells contain swollen rough endoplasmatic reticulum (top right corner), condensed mitochondria (bottom center) and filaments (bottom right corner). The cell-cell contact is developed in the form of typical desmosomes (center). (Original magnification × 12,000.)

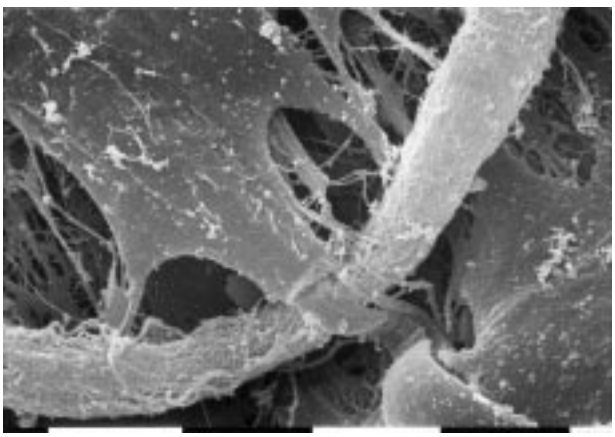

Figure 16 REM micrograph of a fibroblast on the rough aspect of a cross-linked CEM (shrinkage temperature $T_s = 63\,°C$) after a cultivation time of 20 days *in vitro*, partially wrapping around a collagen fiber. (White bar = 10 µm).

elasticity, capacity to take up water and pliability, showed that the chemical treatment did not affect the membranes to an extent, which rendered them not suitable for clinical application. This was true for both degrees of cross-linking ($T_s = 63\,°C$ and $T_s = 81\,°C$).

Since it had been reported that cross-linking can have considerable influence on the biocompatibility of a collagenous matrix [21, 22, 38] we analyzed the growth and spreading behavior of keratinocytes and fibroblasts on this membrane. According to previous results, we did not expect significant toxic effects on cell growth/division, however, it was interesting to know whether cross-linking had an inhibitory effect on cell migration and spreading into the matrix due to the formation of connections between neighboring fibrils. This could be excluded, as we saw especially fibroblasts colonizing the whole thickness of the membrane, with no relevant differences between cross-linked and non-cross-linked membranes.

The results of our *in vitro* studies indicate that this membrane may be a suitable matrix for the permanent replacement of dermis, since its composition and structure offer good conditions for the immigration and colonization by human skin derived cells. Moreover, the mechanical properties of these membranes resemble those of human split thickness skin. Chemical cross-linking of these membranes with carbodiimide can decisively retard enzymatic degradation; it allows – if applied specifically – a controlled degradation of the biomaterial which is adjusted to wound conditions. Furthermore, it offers the advantage of stabilizing the mechanical properties without hampering cell colonization.

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