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A novel native collagen dressing with advantageous properties to promote physiological wound healing

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A novel native collagen dressing with advantageous properties to promote physiological wound healing

Objective: Chronic hard-to-heal wounds generate high costs and resource use in western health systems and are the focus of intense efforts to improve healing outcomes. Here, we introduce a novel native collagen (90 %):alginate (10 %) wound dressing and compare it with the established oxidised dressings

Method: Matrices were analysed by atomic force microscopy (AMF), scanning electron microscopy (SEM), and immunoelectron microscopy for collagen types I, III and V. Viability assays were performed with NIH-3T3 fibroblasts. Matrix metalloproteinase (MMP) binding was analysed, and the effect of the wound dressings on platelet-derived growth factor B homodimer (PDGF-BB) was investigated.

Results: Unlike oxidised regenerated cellulose (ORC)/collagen matrix and ovine forestomach matrix (OFM), the three-dimensional structure of the native collagen matrix (NCM) was found to be analogous to intact, native, dermal collagen. Fibroblasts seeded on the NCM showed exponential growth whereas in ORC/collagen matrix or OFM, very low rates of proliferation were observed after 7 days. MMP sequestration was effective and significant in the NCM. In addition, the NCM was able to significantly stabilise PDGF-BB *in vitro*.

Conclusion: We hypothesise that the observed microstructure of the NCM allows for an effective binding of MMPs and a stabilisation and protection of growth factors and also promotes the ingrowth of dermal fibroblasts, potentially supporting the re commencement of healing in previously recalcitrant wounds.

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chronic wounds • collagen • dressing • extracellular matrix (ECM) • fibroblast • wound healing

Chronic wounds are characterised by a destroyed extracellular matrix (ECM), which is unable to trigger and accelerate regenerative reactions in the wound.[1–4] Therefore, treatment strategies that are designed to replace the absent or dysfunctional ECM may be beneficial.[5]

Collagen is the main structural protein in the human body and is the most abundant protein in the skin.[6] Collagen makes up 80–90 % of the ECM, with proteoglycans, hyaluronic acid and other similar polymers making up the remainder. In recent years there has been renewed interest in collagen-based advanced wound care products[7,8] which, from a theoretical perspective, represent a favourable skin replacement for tissue regeneration.

Over 90 % of the collagen in the body is collagen type I,[9,10] which has a fibrillary form and is known to have around 50 binding partners *in vivo*. The high number of binding partners is presumably required to generate the diversity of fibril patterns, which range from parallel bundles in tendon and ligament, to orthogonal lattices in cornea, and interlocking weaves in blood vessels, skin, and bone.

A collagen-based wound dressing with optimal properties for tissue regeneration has the potential to support ECM replacement and promote cellular ingrowth and matrix remodelling. Application of collagen scaffolds in chronic wounds has demonstrated deposition of new cells, such as fibroblasts; these cells subsequently promote cell growth and tissue formation.[11] The key requirements for a collagen wound dressing are:

- A porous structure, with adequately sized pores that maintain their permeability once the material is applied
- Hydrophilicity that enables adaption to the wound surface and maintenance of fluid uptake like wound exudate or blood
- Surface structures that stimulate ECM deposition, cell proliferation and differentiation
- Ability to bind inflammatory mediators such as matrix metalloproteinases (MMPs).

Many artificial wound-care products do not fulfil all of these criteria; neither do several biomaterials, due to agressive preparation losing the multiple physical and biological properties of the original biological material.

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Chronic or stalled wounds are characterised by a disrupted or damaged ECM that cannot support wound healing. It is widely acknowledged that the ideal properties of a collagen/ECM wound dressing should include a close resemblance to the native ECM structure of the skin.[12] In recent years, commercial collagen-based biomaterials have become widely available for use in wound care. These technologies make use of either reconstituted collagen or collagen purified from tissues as an intact ECM. The function of these wound dressings is to provide a matrix for cell attachment and to facilitate the cellular functions required for successful wound healing.[13] While the mechanism of action of these wound dressings is quite similar, there are differences in key characteristics that substantially affect the performance of the dressing.

Here, we examine the properties of a novel native collagen wound dressing with intact native three-dimensional (3D) collagen fibrous structure and analyse the performance of the native collagen matrix (NCM) *in vitro* compared with the wound dressings oxidised regenerated cellulose (ORC)/collagen matrix and ovine forestomach matrix (OFM) (Table 1). The purpose was to analyse the 3D microstructure and functional properties of an NCM, where the collagen fibrils were retained by a sophisticated preparation process that carefully extracts the collagen from bovine dermis.

Table 1. Overview of characteristics of the three tested products

Feature	Native collagen matrix	Oxidised regenerated cellulose (ORC)/ collagen matrix	Ovine forestomach matrix (OFM)
Composition	Freeze-dried matrix composed of bovine collagen and alginate	Freeze-dried matrix composed of bovine collagen and oxidised regenerated cellulose	Derived sheet from ovine forestomach. Composed of extracellular matrix (ECM)
Composition content	90% Collagen 10% Calcium-alginate	55% Collagen 45% Regenerated oxidised cellulose	90% Collagen 10% Extracellular matrix molecules
Porosity	Yes	Yes	No
Shape	Square	Hexagonal	Square
Fenestration	Two-dimensional	No	One-dimensional
pH wet product	3	3	7
Sterilization	Radiation	Radiation	Radiation
Fluid absorption (g/g)	=20	=20	=5
Shelf life (years)	3	3	3

Fig 1. Atomic force microscopic images of the native collagen matrix (**a**) and human dermis (**b**)

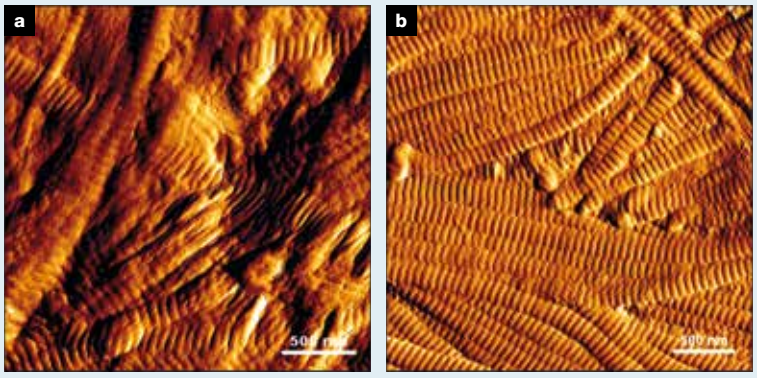
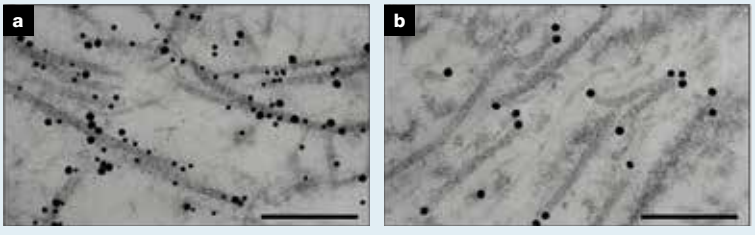


Fig 2. Immunoelectron microscopic images of the native collagen matrix showing type I and type III collagen (**a**) and type V collagen (**b**)



Materials and methods

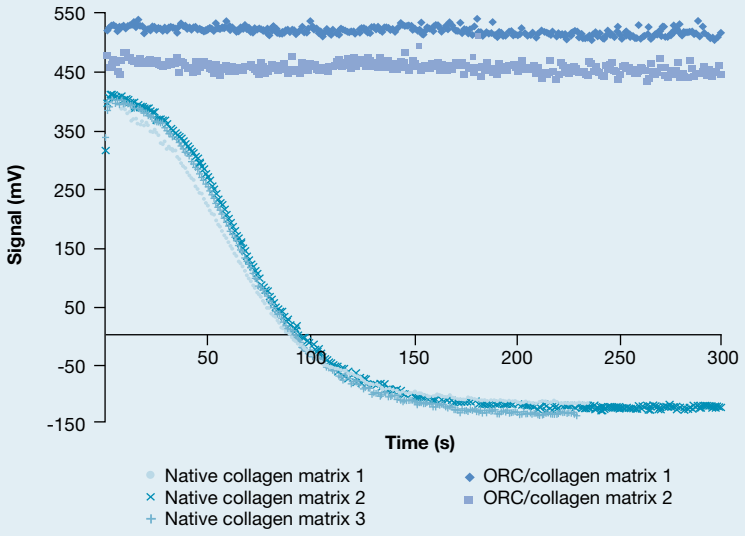
Wound dressings

We compared three different wound dressings: a native collagen matrix (NCM) consisting of 90% bovine collagen and 10% calcium alginate (Cutimed Epiona, BSN medical, Hamburg, Germany); a wound dressing containing collagen and oxidised regenerated cellulose (ORC/collagen matrix, Promogran, Systagenix, Gargrave, UK); and a 10% ECM of ovine forestomach matrix (OFM, Endoform dermal template, Hollister Wound Care, Libertyville, US).

Immunoelectron microscopy of collagen types I, III and V

Samples of the NCM were prepared for ultra thin sectioning and electron microscopy (4°C immediately after the addition of fixative; 2.5% glutaraldehyde in 0.2M sodium cacodylate, pH 7.4). After incubation (room temperature, 1 hour), specimens were dehydrated in ethanol and further processed for epon embedding. Sections were cut with a microtome and mounted on nickel grids. For immunostaining, the grids were floated on top of drops of immune reagents and displayed on a sheet of parafilm. Free aldehyde groups were blocked (50mM glycine) and the grids were incubated in 5% v/v donkey serum and incubation buffer (0.2% acetylated bovine serum albumin in phosphate-buffered saline (PBS); pH7.6, 15 minutes) followed by antigen retrieval with sodium metaperiodate and overnight incubation with primary antibodies (dilution 1:100, 4°C). Specific antibodies against collagen I were labelled with 10nm gold particles; collagen III and V antibodies were labelled with 15nm gold particles. Collagen I and III antibodies were used simultaneously in one experiment, because collagen I and III normally coexist in fibrillary collagen structures. After washing the grids with incubation buffer, they were floated on drops containing gold conjugate reagents (diluted 1/20 in incubation buffer, 60 minutes at room temperature). Sections were post-fixed (2% glutaraldehyde). Finally, sections were washed with distilled water, post-stained with uranyl acetate/lead citrate and examined using a Philips/FEI CM100

Fig 3. Effects of the native collagen matrix and oxidised regenerated cellulose (ORC)/collagen matrix on platelet aggregation *in vitro*



transmission electron microscope with 80 kV accelerating voltage. Images were recorded using a side-mounted Olympus Veleta camera, 2048 x 2048 pixels.

Atomic force microscopy

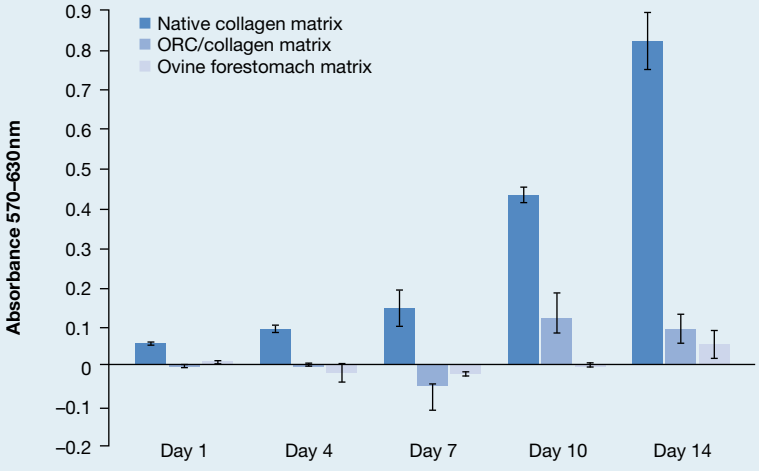
Measurement of samples (1 x 1 cm) of the NCM and OFM was performed in the dry state with an atomic force microscope (NanoWizard 3 JPK Instruments AG, Germany). Tapping mode with a cantilever silicon tip (320 kHz, 42 N/m) was used for image recording.

Platelet aggregation

Platelet aggregation was analysed according to the original test set-up of Born.[14] Porcine blood was treated with sodium citrate (3.2%) and centrifuged (10 minutes, 200 g) and the supernatant, consisting of platelet-rich plasma (PRP), was collected. A circular piece of test material (diameter 8 mm) was added to 1.4 ml of PRP.

Under vigorous stirring the change of optical density (wavelength: 660 nm) of the solution was tracked and recorded for 10 minutes. The change in optical density was expressed in mV.

Fig 5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid assay results for the native collagen matrix, oxidised regenerated cellulose (ORC)/collagen matrix and ovine forestomach matrix measured at days 1, 4, 7, 10 and 14.



Scanning electron microscopy

Samples were fixed, washed with cacodylate buffer and dehydrated with an ascending ethanol series. Specimens were then subjected to critical-point drying with carbon dioxide; absolute ethanol was used as an intermediate solvent.

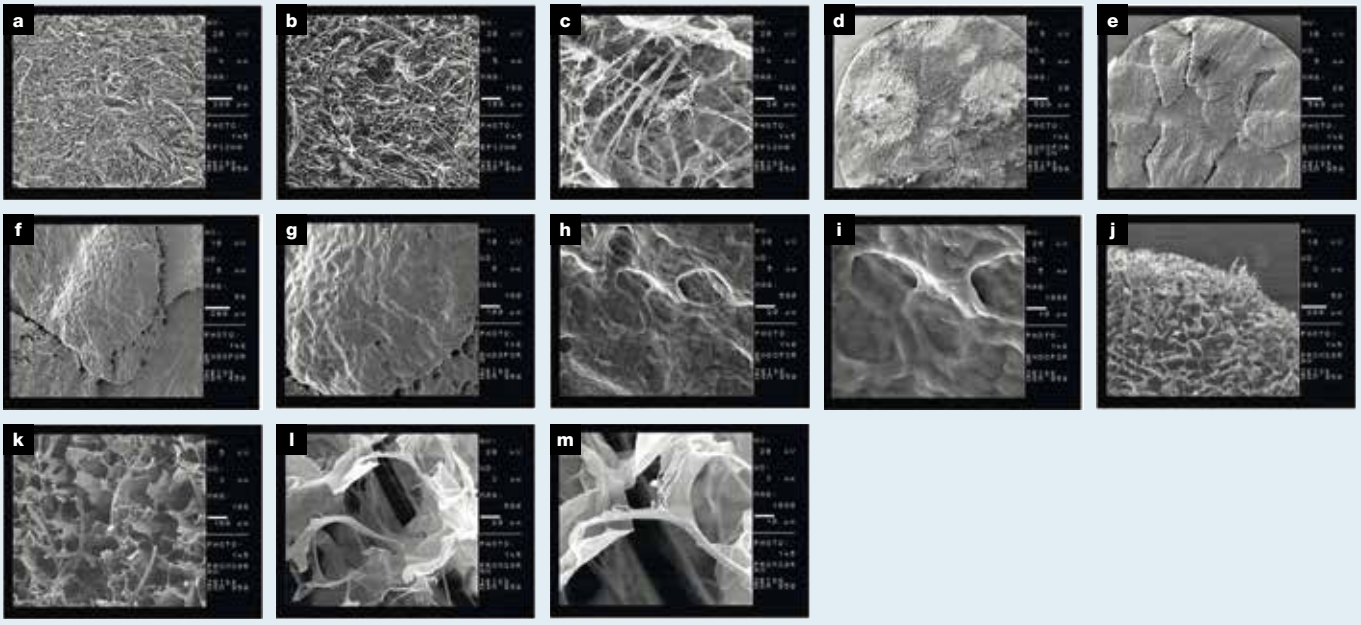
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid assay

NIH 3T3 cells were cultivated in Dulbecco's modified eagle medium (10% neonatal calf serum, 1% penicillin/streptomycin). Cells were seeded on test material (15 mm diameter; 18,000 cells/material) in 200 µl cell suspension and incubated for 1 hour at 37°C to allow adhesion, before 500 µl cell-culture medium was added. Viability assays were performed on days 1, 4, 7, 10 and 14. Medium exchange (700 µl) was performed on days 2, 4, 7, 9, 11 and 13. Medium was removed and cells were washed with warm PBS before adding the working solution (10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) and 90% basal medium) and pipetting into a 24-well-plate. The plate was then incubated for 4 hours at 37°C. Formazan crystals were dissolved out of the cell by adding 600 µl sodium dodecyl sulfate with diluted hydrochloric acid. Absorbance was measured at 570 nm (maximum) and at 630 nm (reference) in a microplate photometer. The final optical density was determined by calculating the difference in the absorbance at 570 nm and 630 nm followed by subtraction of blank values.

Matrix metalloproteinase and platelet-derived growth factor binding assays

Wound dressing samples were cut using 8 mm punch biopsies (Stiefel Laboratorium GmbH, Offenbach am Main, Germany) corresponding to 0.5 cm^2. Subsequently, the samples were placed into 24-well cell-culture plates (Greiner Bio-one, Frickenhausen, Germany). Human MMP-2 and MMP-9 standards (100 ng/ml and 20 ng/ml, respectively), or human platelet-derived growth factor homodimer (PDGF-BB) standard (20 ng/ml) were taken from enzyme-linked immunosorbent assays (ELISAs; R&D Systems GmbH, Wiesbaden, Germany). Reconstituted MMP-2 standard was diluted to a concentration of 5000 pg/ml, the reconstituted MMP-9 standard was diluted to a concentration of 2000 pg/ml and the reconstituted PDGF-BB standard was diluted to a concentration of 1000 pg/ml. Each sample/control was taken in a final volume of 1 ml of protein solution. Samples were incubated for up to 24 hours at 37°C on a plate mixer (THERMOstar, BMG Labtech, Offenburg, Germany). After incubation, supernatants were collected,

Fig 4. Scanning electron microscopic images of the structure of the native collagen matrix (NCM) (**a–c**), ovine forestomach matrix (OFM) (**d-i**) and oxidised regenerated cellulose(ORC)/collagen matrix (**j–m**). The NCM features an open porous structure with web-like structures evident at increasing magnification (**a-c**). In comparison, OFM is bidirectional exhibiting the dense contours of the forestomach villus structure on one side (**d**) and a smooth surface with a small number of pores on the other side (**e-i**). ORC/collagen presents a porous, foam-like structure that appears more amorphous than NCM at higher magnification (**j-m**)



immediately frozen and stored at 20°C until testing. Subsequently, bound PDGF-BB was eluted from the individual wound dressing samples by shaking in 1ml PBS for 1 hour at 37°C. For determination of the MMP-2, MMP-9 or PDGF-BB concentrations the specific ELISAs were purchased from R&D Systems GmbH and run as recommended by the manufacturer.

Tear strength

Tear strength was measured using a Z2.5/TN1S material tester (Zwick, Ulm, Germany). Materials tested were NCM, which is a fenestrated dressing, the non-fenestrated version of the NCM, and an ORC/collagen. Samples were cut to the appropriate size, placed on the machine, fixed and wetted. The system ran with an initial load of 35mm/minute and measured until the material was disrupted. Tear strength was calculated with the following formula:

RSheet = XR mean / XD mean

RSheet—tear strength per sheet in cN/mm; XR mean—tear strength per sheet in cN; XD mean—thickness per sheet in mm.

Statistical analysis

For MMP and PDGF binding assays, independent experiments were carried out twice and measurements were performed in duplicate. Tests to measure the tear strength were carried out at least 9 times for each material. All values are expressed as means ± standard deviation (SD). One-way analysis of variance was carried out to determine statistical significances. Differences are considered statistically significant at a level of $p<0.05$.

Results

Atomic force microscopy (AFM) shows that the NCM (Fig 1a) and its collagen fibrils closely resemble the microstructure of the human dermis (Fig 1b). The regular periodicity in the collagen fibrils structure is observable in both the NCM and human dermis.

To characterise the dressing's collagen composition, immunoelectron microscopy was applied to specifically identify the different types of collagen. Native structures of type I and III collagen (Fig 2a) and type V collagen (Fig 2b) were observed. When comparing the platelet aggregation induced by the NCM and ORC/collagen matrix, only the NCM showed an activation of the clotting cascade visible by a reduction of the optical density over time (Fig 3). ORC/collagen matrix did not trigger any platelet aggregation.

SEM images illustrate that the matrix of the native collagen dressing has an open porous structure with single web-like structures that become obvious at high magnifications (Fig 4 a–c). In contrast to the NCM, OFM has, on one side, a dense contoured matrix in which the forestomach villus structure can be detected (Fig 4 d–f), and on the other side a smooth surface with only few pores (Fig 4 g–i). ORC/collagen matrix also shows a porous, foam-like, structure, although it is more amorphous than NCM (Fig 4 j–m).

Fibroblasts seeded on the NCM showed exponential growth over 14 days, whereas in cells seeded on ORC/collagen matrix or OFM, very low rates of proliferation were observed (only up to maximum 10% of the levels compared with the NCM after 7 days; Fig 5).

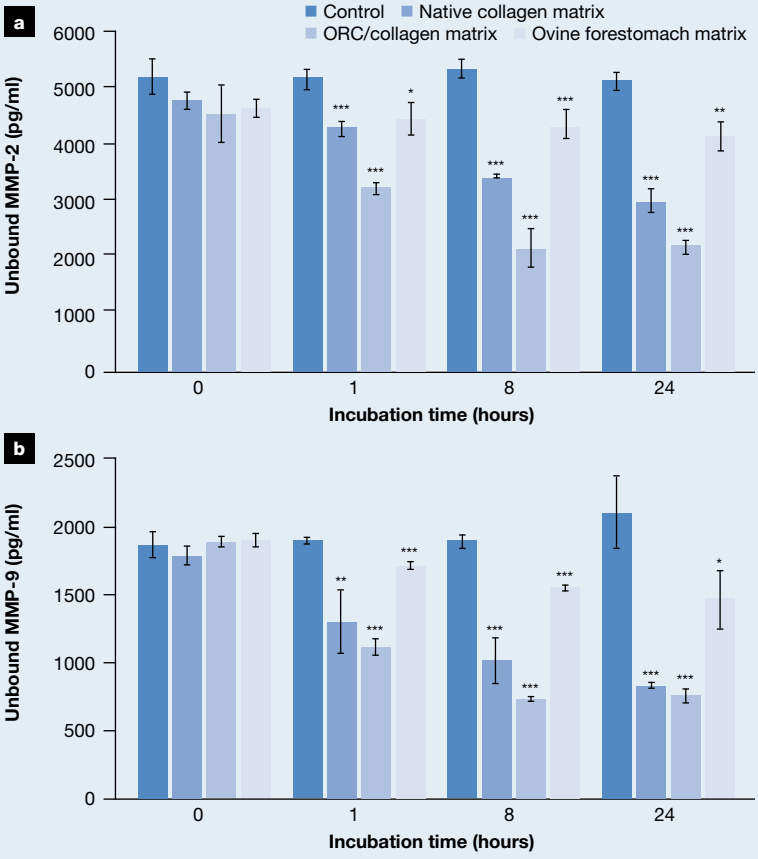


Fig 6. Effects of the native collagen matrix, oxidised regenerated cellulose (ORC)/collagen matrix and ovine forestomach matrix on unbound concentrations of matrix metalloproteinase (MMP)-2 (**a**) and MMP-9 (**b**) (*p<0.05; **p<0.01; ***p<0.001)

The NCM, ORC/collagen matrix, and OFM significantly reduced the MMP-2 concentration in the supernatant (Fig 6a). The NCM exhibited slightly lower binding compared with ORC/collagen matrix, while the binding of the NCM for MMP-2 was significantly higher compared with OFM. Subsequently, only marginal amounts of MMP-2 were detectable in the eluate (data not shown). The dressings also demonstrated a highly significant binding of MMP-9 (Fig 6b). The NCM demonstrated a similar binding as ORC/collagen matrix, over 24 hours. In contrast, binding of MMP-9 by OFM was significantly lower compared with binding by the NCM and ORC/collagen matrix. Again, only marginal amounts of the protease MMP-9 were detected in the eluate (data not shown).

Growth factor assays demonstrated that PDGF-BB is unstable in solution; hence, PDGF-BB concentrations quickly decreased in the controls (Fig 7a). The NCM was able to stabilise significant amounts of PDGF-BB (426.32±7.02 pg/ml after 24 hours) in solution (p<0.001). In contrast, ORC/collagen matrix exhibited only slightly higher PDGF-BB amounts compared with the control after 2 hours, whereas OFM exhibited no protective effect for PDGF-BB *in vitro*.

Subsequently, no PDGF-BB could be detected in eluates from the controls or OFM samples and only marginal amounts of PDGF-BB were detectable in the eluate from ORC/collagen matrix (Fig 7b). Low but significant concentrations of PDGF-BB (11.45±2.75 pg/ml after 8 hours) were released from the NCM (p<0.01).

Non-fenestrated NCM showed a significantly higher tear strength (103±30 cN/mm thickness) compared with ORC/collagen matrix (38±4 cN/mm, p<0.001). Fenestrated NCM also showed a significantly higher tear strength (50±8 cN/mm thickness) than ORC/collagen matrix (38±4 cN/mm, p<0.05) (Fig 8).

Discussion

Immunoelectron microscopy demonstrated that NCM wound dressing is composed of collagen I, III and V. In contrast to the collagen-based wound dressings ORC/collagen matrix and OFM, the NCM has an intact collagen network that closely resembles the microstructure of the human dermis, with AFM images indicating a native structure of collagen fibrils.

The functionality of this native collagen becomes obvious in clotting experiments. It is assumed that a fast and reliable triggering of platelet aggregation indicates that the quaternary structure of collagen is intact.[15–18] When the NCM was incubated with PRP *in vitro* it was shown to effectively induce the clotting cascade, suggesting that the collagen within the dressing has an intact native structure. In contrast, the degraded ORC/collagen matrix was unable to trigger platelet aggregation. Similarly, in a study by Jesty et al. using a similar experimental active platelet aggregation system, no haemostatic effect for the collagen/ORC matrix was observed.[19] This is interesting because both gelatin and ORC have been shown to possess platelet-activating properties. However, an *in vitro* study by Wagner et al. defined a haemostatic activity ranking for different materials: collagen>gelatin>ORC.[20] The differences between NCM and ORC/collagen dressing in inducing platelet aggregation may be due to different experimental procedures and/or due to the lower platelet activation ability of highly processed collagen (gelatin) and ORC.

While collagen-based substrates with an intact microstructure better allow for infiltration and anchorage of cells than degraded structures, pore size also has been shown to have an important role. Collagen dressings have a variety of pore sizes and surface areas. For collagen–glycosaminoglycan scaffolds it was demonstrated that a mean pore size between 20 µm and 120 µm is required to support active skin regeneration processes.[21] Furthermore, fibroblasts and macrophages function best when anchored in a 3D structure, which provides the microarchitecture for cellular infill and thus allows optimal ingrowth of cells.[22,23] SEM demonstrated that the novel NCM has such a 3D structure, with a pore size of approximately 23–56 µm. This is different to the structure of OFM, which has no 3D scaffold and no porosity. In degraded, highly processed collagens, the surface has an amorphous foam-like appearance, as

found in SEM images of the cellular/ORC matrix, indicating animpairment in the collagen structure.

The ECM plays an important role in tissue regeneration and the composition of ECM includes collagen, proteoglycans, hyaluronic acid, fibronectin and elastin. It provides a structural support for cells, while some components of the ECM bind to growth factors to stimulate cell proliferation and migration.[24] Native collagen and collagen fragments control many cellular functions in addition to structural support, including cell differentiation, migration and synthesis of proteins.[25] Fibroblasts, for example, possess specific integrin receptors that recognise intact, native collagen and fibronectin molecules,[26] and it has been shown that native collagen allows for more efficient angiogenesis and greater fibroblast chemotaxis than denatured collagen *in vitro*.[27,28] It is very likely that in strongly processed or degraded collagens the bioactive potential of cells involved in wound healing is lost or at least attenuated. Therefore, a native collagen structure, as demonstrated for the NCM, is advantageous for creating optimal healing conditions in stalled wounds.

As fibroblasts play a key role in wound repair by producing ECM components such as different types of collagen and fibronectin,[29] it is of interest to analyse if a collagen dressing supports fibroblast proliferation *in vitro*. The NCM highly promoted fibroblast cell proliferation. Conversely, fibroblasts seeded on ORC/collagen matrix or OFM showed only very low proliferation levels over 14 days. It is worth noting that, in a study using a cell infiltration assay based on 4',6-diamidino-2-phenylindole staining, fibroblasts seeded on OFM were able to bind and proliferate in the matrix.[30] We postulate that the native 3D collagen structure of the NCM provides the optimal microarchitecture for cellular infill and thus allows ingrowth of cells, which could be advantageous in the treatment of chronic wounds.

MMPs play a major role in the degradation of native as well as partly degraded fragments of collagen. Dynamic collagen formation and degradation processes are important events in acute wound healing. However, MMP levels are known to be elevated in chronic wounds,[31] which can result in 'off-target' destruction of proteins essential for wound healing, such as growth factors and ECM proteins. Here, we demonstrated that the NCM and ORC/collagen matrix were found to bind to MMP-2 and MMP-9 to a similar extent; the binding by OFM was significantly lower. In addition, further studies shall evaluate the effect on activity of these proteases, which has been shown to be of interest.[32,33]

Previous studies have shown that collagen dressings may bind growth factors like PDGF-BB and protect them from degradation and enzymatic cleavage.[34] The binding is most certainly promoted by non-covalent interactions[35] and might differ depending on such factors as the origin of the collagen, the treatment of the collagen during production, and the sterilisation processes involved.[36] The NCM was able to significantly stabilise PDGF-BB *in vitro*, whereas only slight effects of ORC/collagen matrix were observed, while OFM exhibited no influence. Additionally, the NCM was able to release low but significant amounts of PDGF-BB after the incubation. However, binding of PDGF-BB by the NCM appears to be

Fig 7. Effect of the native collagen matrix, oxidised regenerated cellulose (ORC)/collagen matrix, and ovine forestomach matrix (OFM) on platelet-derived growth factor B homodimer (PDGF-BB) in solution (**a**). Release of PDGF-BB from the native collagen matrix, ORC/collagen matrix, and OFM dressing samples into the eluate (**b**). (*p<0.05; **p<0.01; ***p<0.001)

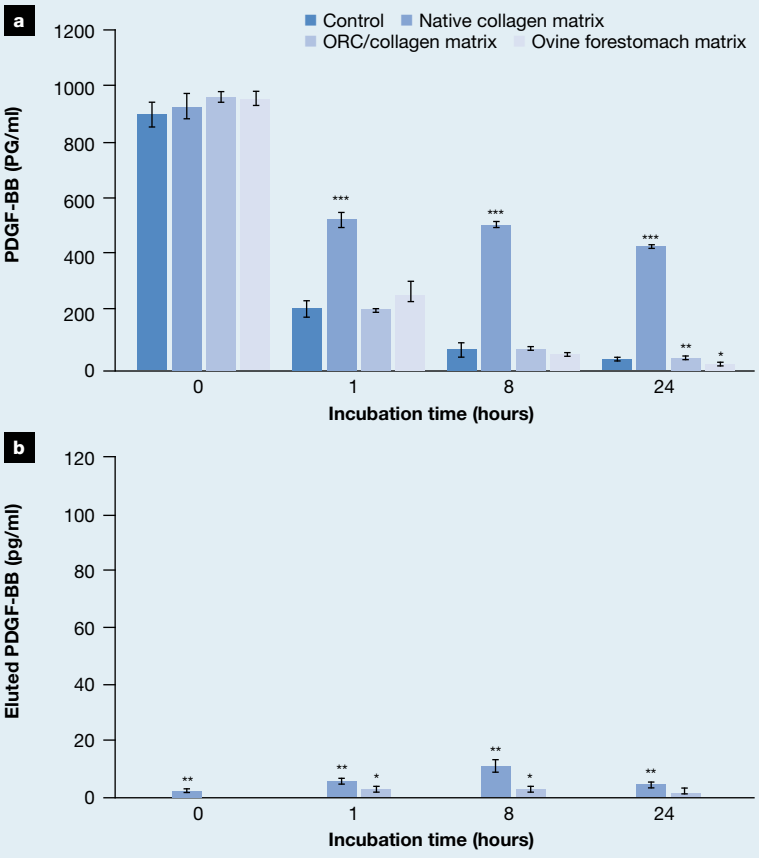
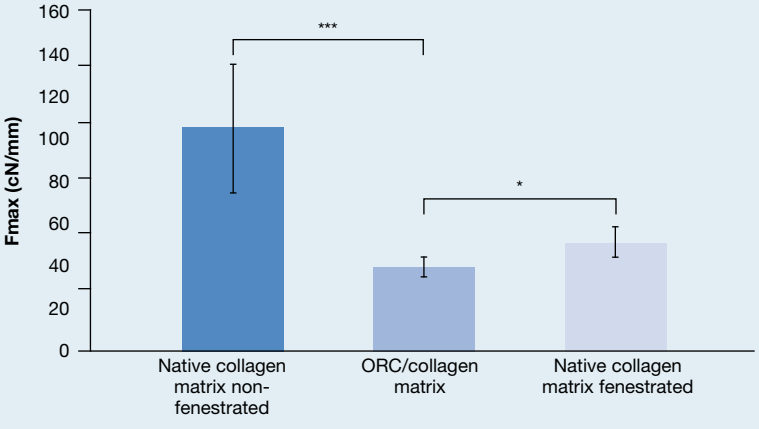


Fig 8. Tear strength analysis of the native collagen matrix (fenestrated versus non-fenestrated) compared with oxidised regenerated cellulose (ORC)/collagen matrix. (*p<0.05; **p<0.01; ***p<0.001)



considerably weaker than binding of proteases like MMP-2 and MMP-9. The binding of proteases may result in the observed stabilisation of the growth factor in the incubation solution by reducing their proteolytic degradation. Hence, it can be speculated that the NCM may exhibit beneficial effects in the treatment of chronic wounds with a high proteolytic activity and an excessive inflammatory response by protection of growth factors such as PDGF-BB. We hypothesise that the MMP binding capability of the NCM, combined with its ability to stabilise growth factors and to promote cell proliferation, is likely to allow for better tissue remodelling compared with ORC/collagen matrix or OFM-based wound dressings. It should be kept in mind that the data presented here are *in vitro* data and cannot be translated directly to clinical practice. Thus, to test this hypothesis, clinical data are needed.

With regard to clinical practice, an important requirement for a wound dressing is ease of handling and application by the user. Therefore, we tested the tearing strength of the different dressings. Fenestrated NCM displayed competitive tearing strength compared with non-fenestrated collagen/ORC matrix, showing that the combination of calcium alginate and native collagen enhances product stability. We postulate the preparation process retains the native structure of the collagen fibrils, the stability of the wet product is enhanced.The study was done *in vitro*, hence, direct translation of results into the clinical setting is limited.

Conclusion

In conclusion, our evaluation of a novel NCM reveals that this wound dressing is characterised by a native collagen structure that supports the biomimetic strategy of an ECM replacement by promoting cellular ingrowth and matrix decomposition into the wound. It demonstrated an *in vitro* stability that can act as a scaffold for dermal fibroblasts and form a sufficiently resilient bioactive matrix to reduce the frequency of dressing changes with the intention of optimising the wound healing over a more balanced treatment period. The optimised tear strength and fenestrated structure of the NCM has the potential to be practical in the clinical setting. Studies in a clinical setting are underway to test the observed *in vitro* properties. **JWC**

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