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ARTICLE

Evaluation of Dense Collagen Matrices as Medicated Wound Dressing for the Treatment of Cutaneous Chronic Wounds

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Cutaneous chronic wounds are characterized by an impaired wound healing which may lead to infection and amputation. When current treatments are not effective enough, the application of wound dressings is required. To date, no ideal biomaterial is available. In this study, highly dense collagen matrices have been evaluated as novel medicated wound dressings for the treatment of chronic wounds. For this purpose, the structure, mechanical properties, swelling ability and in vivo stability of matrices concentrated from 5 to 40 mg/mL were tested. The matrix stiffness increased with the collagen concentration and was associated with the fibril density and thickness. Increased collagen concentration also enhanced the material resistance against accelerated digestion by collagenase. After subcutaneous implantation in rats, dense collagen matrices exhibited high stability without any degradation after 15 days. The absence of macrophages and neutrophils evidenced their biocompatibility. Subsequently, dense matrices at 40 mg/mL were evaluated as drug delivery system for ampicillin release. More concentrated matrices exhibited the best swelling abilities and could absorb 20 times their dry weight in water, allowing for an efficient antibiotic loading from their dried form. They released efficient doses of antibiotics that inhibited the bacterial growth of *Staphylococcus Aureus* over 3 days. In parallel, they show no cytotoxicity towards human fibroblasts. These results show that dense collagen matrices are promising materials to develop medicated wound dressings for the treatment of chronic wounds.

1. Introduction

Cutaneous chronic wounds are characterized by the absence of healing after 6 weeks. More than 6 million people are affected by these pathologies in the United States of America [1]. The most prevalent cutaneous chronic wounds are venous ulcers, diabetic foot ulcers and bedsore [1-2]. The number of affected persons is expected to increase in the next years as the American population ages and more people develop diabetes [3]. Ulcers increase the risk of infection, which sometimes leads to the leg amputation [4-5] . Impaired skin wound healing is characterized by a chronic inflammation, an extracellular matrix breakdown and an impaired re-epithelialization [2]. The classic treatment is the debridement of the wound followed by its compression with sterile gauze [6-7].

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When this method is not effective enough, chronic wounds have to be dressed with adequate biomaterials to protect the long-term healing from infection [6, 8]. The ideal wound dressing should absorb exudates, create a moist environment and promote wound healing [9]. In addition, these biomaterials should be biocompatible and removed from wounds without trauma. Last, thermal insulation and gaseous exchanges must be effective through the wound dressing. To date, no dressing is effective for all types and stages of chronic wounds [9-10]. Nowadays, research orientation is towards "bioactive dressings" that can play an active role in the wound healing process [6]. The first strategy is based on the application of skin substitutes, following a cell therapy approach. These materials consist of a scaffold in which living cells are incorporated and can produce a panel of cytokines promoting tissue repair [4, 11]. However, skin substitutes are expensive and difficult to fabricate, with limited self-life [6]. The second strategy relies on the wound coverage by medicated dressings. These modern dressings aim to deliver biomolecules such as antibiotics and growth factors in a prolonged manner [6, 8].

Collagen-based materials can be considered as ideal biomaterials for wound dressing application owing to the intrinsic properties of collagen I. *In vivo*, this natural and biodegradable protein provides strength and resistance to connective tissues. In addition, collagen I is a natural ligand

of cells [12]. Collagen-based biomaterials can be obtained from the decellularization of native tissues [13-14]. These materials have the native structure of their original extracellular matrices (ECM) but can be immunogenic if the decellularization process is not effective [15]. An alternative strategy is based on the utilization of collagen I as an elementary brick to build-up 3D matrices. Among them, sponges and hydrogels are the most studied. Collagen sponges are produced by freeze-drying of Atelocollagen I solutions to generate dry scaffolds. These acellular materials are cross-linked by chemical reagents to improve their stability at physiological temperature. Unfortunately, cross-linkers such as glutaraldehyde are toxic *in vivo* [16]. In addition, sponges are poor drug delivery systems because of their open-pore geometry [17]. This explains why they are not used as medicated wound dressing to treat chronic wounds. Uncross-linked collagen hydrogels obtained via neutralization of type I collagen acidic solutions have also shown great promises in this area [17]. However they suffer from weak mechanical properties that limit their utilization as biomaterials. They are only used after hydrogel contraction by fibroblasts [18-19] .

Dense collagen matrices have been developed over 10 years in order to correct the drawbacks of collagen hydrogels and sponges. These biomaterials are fabricated by gelation under ammonia vapors of collagen solutions concentrated up to 40 $mg.mL^{-1}$ [20]. These acellular materials are easy to produce and cost-effective. In addition, they do not require chemical cross-linking. An *in vitro* study has revealed that fibroblasts seeded on the surface of 40 $mg.mL^{-1}$ matrices were able to colonize them up to 300 µm in depth, but this process was slow [21-22]. In contrast, 5 $mg.mL^{-1}$ matrices were fully colonized but 50% of the materials were degraded after 15 days in culture.

In this study, dense collagen matrices have been evaluated as novel medicated wound dressings for the treatment of cutaneous chronic wounds. We hypothesized that the high collagen concentration could improve the structure, mechanical properties and biological stability of these matrices and, ultimately, should be beneficial for their drug release properties. For this purpose, the influence of collagen concentration on the physical properties has been studied *in vitro*. In addition, the stability and the biocompatibility of collagen matrices have been evaluated *in vivo* in rats after subcutaneous implantation. Last, the ability of the densest materials to uptake and release an antibiotic has been investigated in detail.

2. Materials and Methods

2.1 Preparation of dense collagen matrices

Type I collagen was extracted from rat tail tendons in a 0.5 M acetic acid solution and adjusted to a concentration of 5 $mg.mL^{-1}$ as previously described [21]. Then, the collagen solution was progressively concentrated up to 10, 20 and 40 $mg.mL^{-1}$ by slow evaporation of the solvent under laminar flow bench in sterile conditions. The collagen concentration was assessed by hydroxyproline titration**.** To prepare dense collagen matrices, 1 mL of 5, 10, 20 or 40 $mg.mL^{-1}$ collagen solutions was poured into each well of a 24 well plastic plate and gelled by placing the samples for 24 hours under concentrated ammonia vapors. Last, dense collagen matrices were rinsed with sterile PBS for 3 days until the pH was stabilized at 7.4. For rheological measurements, 5 ml of collagen solutions were poured into plastic dishes and processed as previously described to generate discs with a 5 cm diameter. Regarding the molding experiments, molds were filled up to get a flat surface at the top part.

2.2 Rheological measurements

Shear oscillatory measurements on collagen matrix discs were performed on a Bohlin Gemini rheometer (Malvern) fitted with a plan acrylic 40 mm diameter geometry. Both base and geometry surfaces were rough in order to avoid sample slipping during measurement. All tests were performed at 37 °C. Mechanical spectra, namely storage, G′ and loss, G″ moduli versus frequency, were recorded at an imposed 1% strain, which corresponded to non-destructive conditions, as previously checked (data not shown). In order to test all collagen matrices in similar conditions, before each run, the gap between base and geometry was chosen so that a slight positive normal force was applied on gels during measurement. Four samples of each matrix type were tested.

2.3 Scanning electron microscopy (SEM) analysis

Collagen matrices were fixed using 3.63% glutaraldehyde in a cacodylate/saccharose buffer (0.05 M/0.3 M, pH 7.4) for 1 hour at 4 °C. Following fixation, samples were washed three times in a cacaodylate/saccharose buffer (0.05 M/0.3 M, pH 7.4) and dehydrated through successive increasing concentration ethanol baths from 70% to 100% alcohol. Thereafter, samples were dried in a critical point dryer and gold sputtered (20 nm) for analysis. Samples were observed with Hitachi S-3400N SEM operating at 10 kV

2.4 Transmission electron microscopy (TEM) analysis

Samples were fixed as described above. Following fixation and washing, samples were post-fixed using 2% osmium tetra-oxide in a cacodylate/saccharose buffer (0.05 M/0.3 M, pH 7.4) for 1 hour at 4 °C. Samples were then washed three times in a cacodylate/saccharose buffer, dehydrated with ethanol and embedded in araldite. Thin araldite transverse sections (100–200 nm) were performed by an Ultracut ultramicrotome (Reichert, France) and contrasted by phosphotungstic acid. Slides were then analysed with a Cryomicroscope Tecnai spirit G2 electron microscope operating at 120 kV. For each matrix, ten photos were taken at magnification X15000 and analysed. The diameter of each fibril was measured on the numerical images. Three matrices for each collagen concentration were analysed. The results were expressed as the mean +/- standard deviation (SD).

2.5 Suture retention test

To investigate the resistance of dense collagen matrices to tearing during the suture procedure, a study of tensile strength

at the breaking point was performed. Samples of collagen from 5 to 40 mg.mL^{-1}, 2.5 cm x 2cm size and 5mm thick (n=5) were fixed to a grip. Each sample was cut into two parts. A polypropylene suture (Prolene© 6-0, Ethicon, France) was performed at 0.5 cm from the edge of both parts. Tensile tests were performed at a speed of 9 mm/min up to the breaking point (MTS Bionix 858 Mini 2 Universal Test Machine).The force at the breaking point of sutures was then measured. Results were reported as mean ± SD.

2.6 *In vivo* collagen matrix implantation

2.6.1. Surgical procedure

The procedure and the animal treatment complied with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research. The studies were carried out under authorization no. 006235 of the Ministère de l'Agriculture, France. Twelve adult Wistar male rats weighing 250 g (Wi/Wi, Charles-Rivers France) were anaesthetized by intraperitoneal injection of sodium pentobarbital solution (30 mg/kg, Centravet France). The abdomen was shaved and disinfected. A vertical incision was made on the abdominal midline, and 1 cm^2 collagen matrices at 5, 10, 20 and 40 mg.mL^{-1} were implanted in a subcutaneous pocket (n = 12). The skin and the muscle layer were then sutured (Vicryl® 4/0). After 15 and 30 days post-surgery, the rats were euthanized by intraperitoneal injection of sodium pentobarbital (60 mg.kg^{-1}). The collagen matrices were then collected and fixed in 4% paraformaldehyde (Merck France) in PBS for 24 hours, dehydrated and embedded in paraffin. For each condition, three matrices were processed for histological analyses.

2.6.2. Histological analysis and indirect immunodetection

Ten micro-meter-thick sections, transverse to the matrix surface were performed with a manual microtome (Staissnie France). Paraffin sections were then rehydrated and stained with hemalum-eosin. The sections were dehydrated, mounted between slide and coverslip and observed with an optical microscope (Nikon E600 POL). Photos of the collagen matrix slides were taken with a CCD camera (Nikon). Macrophages were detected by indirect immunodetection using a CD-68 antibody. After rehydration and an extended wash in phosphate-buffered saline solution (PBS), the rehydrated sections were incubated for 5 min at room temperature with 0.2% pepsin in acetic acid 10% (v/v). The sections were rinsed in PBS and incubated for 30 min in PBS containing 1% (w/v) glycine. After another wash in PBS, the sections were incubated for 60 min at room temperature with a blocking solution (0.05% tween PBS, 1% bovine serum albumin, 10% calf serum). A primary antibody against CD-68 (AbD Serotec, France) diluted 1/100 (v/v) was added and slides were placed in a moist chamber overnight. The following day, sections were incubated in moist, dark chamber for 90 min with a secondary antibody anti-mouse coupled with Rhodamine diluted 1/400 (v/v) (Molecular Probe). After three rinses in PBS, sections were incubated for 10 min in a DAPI bath (dilution: 1/50,000 v/v). Last, the slides were rinsed three times in PBS, mounted between slide and coverslip and observed with a fluorescence microscope AXIO 100 (Zeiss).
Neutrophils were identified by naphthol AS-D chloroacetate esterase staining (Sigma-Aldrich). Briefly, rehydrated sections were incubated with a napthtol AS-D cloracetate solution for 15 minutes in a dark chamber. The slides were rinsed in water and counterstained with hemalum-eosin for two minutes. Finally, the sections were then washed three times for 5 minutes in water and air dried. Slides were observed with an optical microscope as previously described.

2.7 Swelling properties

Prior to lyophilization, each collagen matrix was weighed after removal of excess fluid. Then, samples were freeze-dried using a Lyophilizator and weighed. Last, dried collagen matrices were immersed in PBS at 25°C. At different time points (1, 4, 7 and 11 days), matrix weight was measured and the corresponding swelling ratio calculated using the following equation (eq. 1):

$$\text{Swelling ratio (\%)} = \frac{W_{rh} - W_d}{W_d} \times 100 \qquad (1)$$

with W_d being the dried weight of the matrix and W_{rh} its rehydrated weight**.**
The swelling ability of the matrices was also calculated as the ratio of the weight W_{rh} of the rehydrated form divided by W_{ih} the weight of the as-synthesized hydrated form (i.e. before freeze-drying).(eq. 2):

$$\text{Swelling ability (\%)} = (W_{rh} / W_{ih}) \times 100$$

2.8 Accelerated collagenase digestion.

Accelerated collagenase degradation of collagen hydrogels was studied in the presence of collagenase type I (Sigma, 125 CDU.mg^{-1}) using a solution at 30 U.mL^{-1} in PBS. One gram of dried collagen matrices was incubated in 2 mL of collagenase solution at 37 ºC. At specific time intervals, 100 µL was collected and replaced by fresh collagenase. Then, hydroxyproline titration was performed to assess the quantity of degraded collagen within samples. Last, the degraded collagen fraction was expressed as a percentage of the initial quantity of collagen hydrogel.

2.9 Ampicillin loading

CM40s were prepared and freeze-dried as previously described (section 2.7). In this experiment, 100 µL of 40 mg.mL^{-1}collagen solution was used to form either matrix. Samples were weighted before and after drying. Then, dried CM40s were incubated for 24 hours in 3 mL of an ampicillin solution concentrated at 2 mg.mL^{-1} (CM40Ampi2), 20 mg.mL^{-1} (CM40Ampi20) or 200 mg.mL^{-1} (CM40Ampi200). Last, ampicillin-loaded matrices were freeze-dried and weighted. The drug loading was measured by the difference between the matrix weight after and before impregnation with

ampicillin. The initial drug content was also checked using the cumulative dose of ampicillin at the end of the drug release. Control samples were performed by impregnation of dried CM40s with PBS and processed as previously described.

2.10 Antibacterial activity monitoring

Staphylococcus aureus (ATCC 29213) were incubated overnight at 37°C in Luria-Bertani (LB) broth (yeast extract, 5 $g.L^{-1}$; NaCl, 10 $g.L^{-1}$ and triptone, 10 $g.L^{-1}$). LB Agar was used throughout this experiment as the growth media to measure the inhibitory effect of ampicillin on bacteria. Antibiograms were carried out using a diffusion method from collagen matrices. Briefly, a bacterial suspension (culture dilution 1:100) was spread on an agar petri dish, and incubated for 10 minutes at room temperature. Then, Freeze-dried ampicillin-loaded collagen matrices (CM40Ampi2, CM40Ampi20 and CM40Ampi200) were set onto the petri dishes. Bacteria were then allowed to grow for 24 hours at 37 °C. The inhibitory effect of antibiotics on bacteria was determined by measuring the diameter of the bacteria-free zones surrounding the matrices. In parallel, a standard curve was performed by dropping 10 µL of antibiotic solutions on paper discs of increasing concentrations of ampicillin (0.1-5.0 $mg.L^{-1}$) and by measuring the diameter of the inhibition zone. To analyze the antibiotic release from ampicillin-loaded collagen matrices over 4 days, the materials were transferred from a petri dish to a new one on a daily basis. Thanks to the standard curve, released doses were daily calculated and expressed as a function of time. In all cases, results were expressed as mean $\pm$ SD (n=6). Lastly, quantities were expressed as an ampicillin concentration by dividing the released dose over the matrix volume and compared with Minimum Inhibitory Concentration (MIC) of ampicillin for *S.Aureus*. For this purpose, MIC was determined following the dilution method as previously described.

2.11. Cytotoxicity experiments

2.11.1. Cell culture

Primary dermal fibroblasts were grown in adherent culture flasks in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Streptomycin. Cells were kept at 37º C in a humidified 5% carbon dioxide chamber until confluence was reached. Harvesting was done with a trypsin-EDTA solution. Before each use, cells were stained with a 1% trypan blue solution and counted with a Malassez hematocytometer.

2.11.2. Viability tests

Primary fibroblasts were seeded into 6 well plates at the density $1x10^5$ per well. After a six hour culture period, ampicillin loaded-CM40s (CM40Ampi2, CM40Ampi20 and CM40Ampi200) were added into wells. Cell toxicity of CM40Ampi2, CM40Ampi20 and CM40Ampi200 was assessed over 6 days. Cell metabolic activity was measured at day 1 and 6 using an Alamar Blue assay ® . Medium was first removed and rinsed three times with 500 µL of colorless DMEM supplemented with 10% serum. Then, 50 µL of Alamar Blue stock solution (Life technologies) was added into the culture medium. Samples were incubated in a humidified 5% carbon dioxide chamber for 4 hours. Subsequently, media were removed and diluted 1 in 2 in colorless DMEM. The absorbance was recorded at 570 nm (oxidized resazurin) and 600nm (reduced resazurin). Control samples were cells incubated with unloaded CM40s and cells without CM40s. For each sample, percentage of dye reduction was calculated following the formula provided by the supplier. Last, results were expressed as a percentage of the control (cells alone without CM40s). In all cases, results were expressed as mean $\pm$ SD (n=6).

2.12. Statistical analysis

All experiments were carried out at least twice and the results were expressed as the mean values ± standard deviation (SD). In all cases, data are means $\pm$ SD. The differences were analyzed using one way ANOVA, followed by Tukey post-test, $p < 0.05$ was considered significant. Statistical analysis was carried out between CM40 and other collagen matrices for rheological analysis, swelling analysis, suturation test and degradation analysis.

3. Results

3.1 Morphological and mechanical characterization of dense collagen matrices

Collagen Matrices (CMs) prepared by neutralization of collagen acidic solutions under ammonia vapours showed increasing opacity as the initial collagen concentration increased (Figure 1A). CM20 and CM40 were obtained as stiff materials whereas CM5 and CM10 appeared softer. Nevertheless, all CMs were easy to handle. Using different molds, it was possible to recover dense collagen matrices whose shapes nicely preserve the imprint of their initial container (Supporting information 1). Mechanical properties of dense CMs were investigated by rheological measurements (Figure 1B). Storage, G' and loss, G'' moduli were measured versus frequency and showed no significant variation with this parameter in the 0.1-10 Hz domain (data not shown). For each matrix, G' was more than one decade higher than G" indicative of their predominant elastic behaviour. Both moduli increased according to the collagen concentration from CM5 to CM40 (Figure 1B). The elastic moduli ranged from 1 to 10 kPa. CM40 was about 10 times stiffer than CM5 ($p < 0.05$). G' for CM20 was three times higher than for CM5. Lastly, G' measured for CM10 was similar to that of CM5.

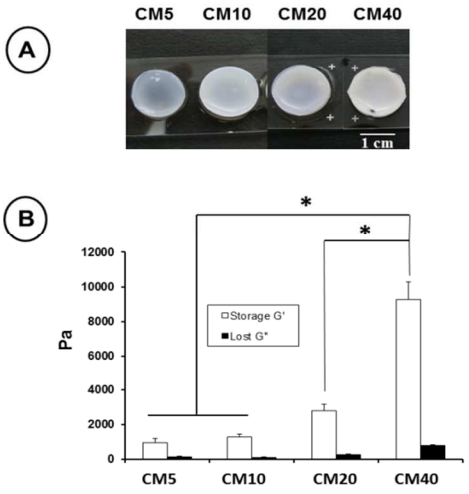

Figure 1: Physical properties of collagen matrices (CMs). (A): Macroscopic view of dense collagen matrices. (B): Rheological properties of dense CMs. (*: $p<0.05$, Anova one way, post-test: Tukey).

3.2 Structural characterization

Observation by SEM showed the presence of a fibrillar network within dense collagen matrices regardless of their concentration. CM5 was characterized by a large porosity (Supporting information 2). When the concentration increased up to 20 $mg.mL^{-1}$, the porosity seemed to decrease. In CM40, bundles of fibrils were visible.

Further investigation by TEM provided structural details of dense collagen matrices. Cross-striated fibrils (with a 67 nm period) were visible in either type of dense CMs. Nevertheless, density, homogeneity and fibril diameters were different according to the collagen concentration. The analysis revealed the presence of thin fibrils in CM5 and CM10 (Figure 2).

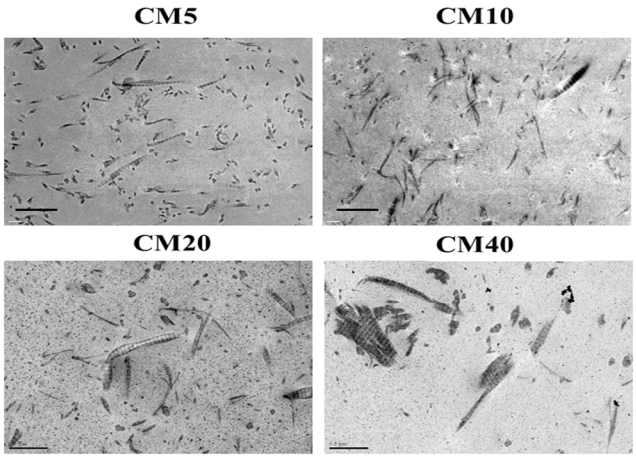

Figure 2: TEM images of dense collagen matrices. Bar 500 nm.

In these matrices, the fibril population was homogenous, *i.e* around 80% of fibrils were less than 50 nm in diameter (Table 1). When the collagen concentration increased, a significant change in fibril morphology was observed with the presence of thick fibrils (Figure 2). They represent ca. 25-30 % of the fibrils population in CM40 (Table 1).

Table 1: Diameters of fibrils within collagen matrices (% of total)

Collagen Matrix	0-50 nm	50-100 nm	100-200 nm
CM5	77.8 ± 14.2	20.6 ± 12.0	1.6 ± 2.3
CM10	85.1 ± 9.9	14.0 ± 10.5	0.8 ± 1.4
CM20	62.0 ± 10.0	27.8 ± 15.8	10.2 ± 5.8
CM40	32.8 ± 6.2	40.0 ± 4.2	27.2 ± 8.8

3.3 Suture retention strength of dense collagen matrices

In order to assess the performance of collagen matrices (CMs) for clinical implantations, the resistance of these materials to forces applied at the suture point was measured for either type of CMs. A macroscopic view of CMs demonstrated the best performance of CM40 (Figure 3A) whereas CM5 was more sticky during the suture process. The analysis showed that the peak load value increased proportionally with the collagen concentration (Figure 3B). The suture retention strength reached about one Newton in CM40, *i.e* 10 times higher than that measured for CM5 ($P<0.05$).

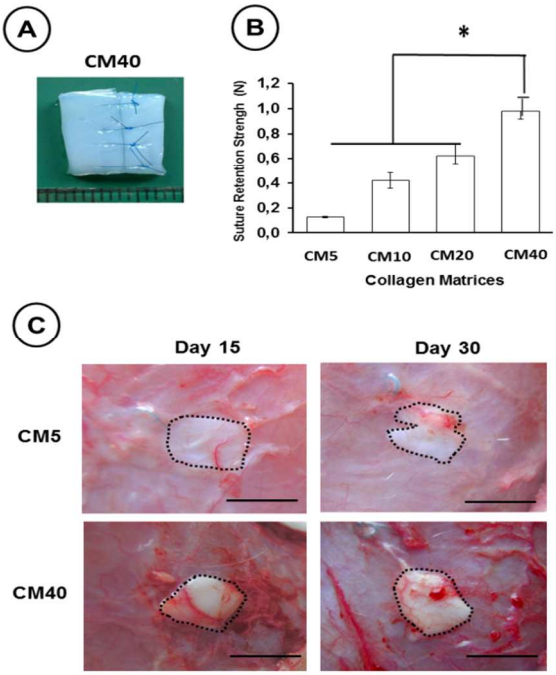

Figure 3: Suture retention strength and in vivo stability of dense collagen matrices. (A): Macroscopic view of CM40 after suture of the matrix half sections. (B): Suture retention strength of matrices with regard to their collagen concentration. (*: $p<0.05$, One way Anova, post-test: Tukey). (C): Macroscopic view of dense collagen matrices 15 and 30 days after implantation in rats. Dotted lines surround dense collagen matrices. Bar: 1 cm.

3.4 *In vivo* stability and biocompatibility

Collagen matrices were implanted in subcutaneous pockets with the aim to evaluate their stability *in vivo* and their

biocompatibility. After 15 and 30 days post-surgery, the macroscopic view of implants revealed the absence of severe foreign body response. No macroscopic inflammation (redness, edema) and fibrous cap were visible (Figure 3C). After 15 days, CM5 appeared flat without well-defined edges. After one month, CM5 were barely visible and a fraction of their original area had disappeared. The histological analysis showed a complete colonization of CM5 by host cells at day 15 (Figure 4). The cell colonization was associated with a reduction of matrix thickness (by 70%) evidencing the remodelling of these materials.

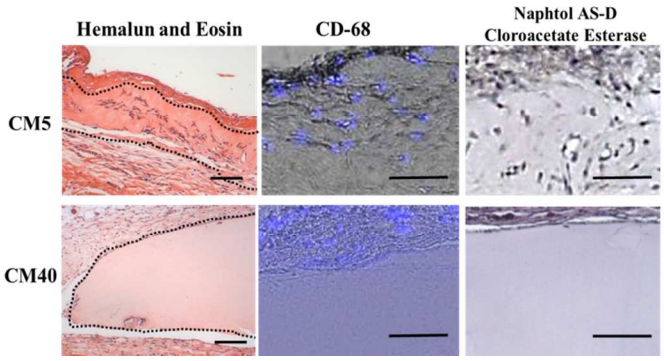

Figure 4: Tissue integration and biocompatibility of collagen matrices. Complete colonization of CM5 by host cells from day 15 after implantation. In contrast, no cellular colonization was observed in CM40 at day 15. Dotted lines surround dense collagen matrices. No macrophages detected within implants at day 15 (CD-68 immunodetection). Cells nuclei labelled with by DAPI (blue). No neutrophils detected by labeling of the AS-D chloroacetate esterase. Cells nuclei labelled with hemalun (blue). Bar: 200 µm.

As a comparison, CM40 appeared stuck onto the tissue surface, just held by a thin conjunctive membrane (Figure 3C). The analysis of histological sections revealed the absence of cell colonization at day 15 (Figure 4). At this time, the matrix thickness was close to the original one, *i.e* 1 mm. When the colonization of CM40 was analysed after 30 days post-surgery, cell infiltration was visible over 300 µm depth (Supporting information 3).

The presence of neutrophils was assessed by detection of AS-D chloroacetate esterase. This type of cells was not detected (brown staining) within collagen matrices regardless of their concentration (Figure 4). CD68 is a specific marker allowing the detection of macrophages. After immunodetection on histological sections, no CD68 positive cells were detected (red fluorescence) regardless of the collagen concentration. Only cell nuclei were visible (blue fluorescence). As a result, this observation evidenced the absence of severe or chronic inflammation after implantation of collagen matrices.

3.5 Swelling properties of dense collagen matrices

Starting from the same volume (1 mL) of collagen solution, no variation was observed in the dimensions of the final hydrated matrices as a function of collagen concentration and no visible contraction occurred during gelation. (Figure 5A). After freeze-drying, the matrix volume slightly decreased regardless of the matrix type. When the dried forms of CM5 and CM10 were incubated in PBS, a contraction phenomenon was observed within a few seconds. In contrast, the volume of CM20 and CM40 remained unchanged immediately after water addition.

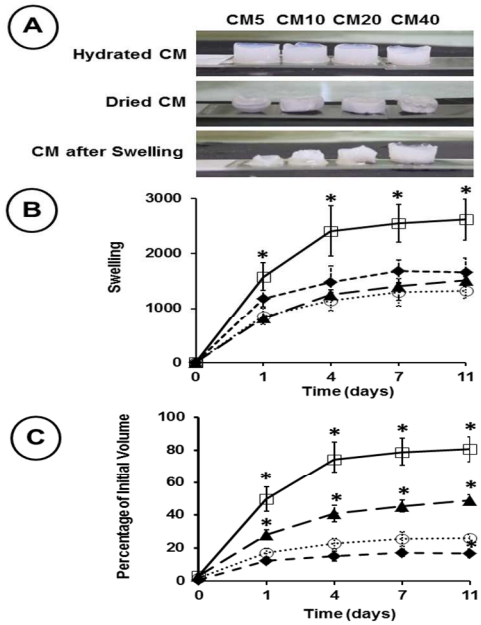

Figure 5: Swelling properties of dense collagen matrices. (A): Macroscopic view of collagen matrices in their hydrated form, after freeze-drying and after rehydration. (B): Swelling ratios of dense CMs. (*: $p<0.05$, Anova one way, post-test:Tukey). (C): Percentage of the initial volume of dense CMs after swelling. (◯ - CM5, ◆ - CM10, ▲ - CM20, □ - CM40).

An important swelling was observed for all collagen matrices during the first day of water absorption. At this time point, the swelling ratio calculated in CM40 was around 1600 whereas it was only about 1000 in the other matrices (Figure 5B). From day 1, the swelling ratio sharply increased in CM40 to reach ca. 2500 at day 4. Regarding the other matrices, this ratio slightly increased to reach a plateau value around 1300 at day 4. As reference cross-linked collagen sponges concentrated at 5 $mg.mL^{-1}$ showed a much smaller swelling ratio (ca. 800) (Supporting information 4).

The swelling ability was also expressed as a percentage of the initial volume of the dense matrices (Figure 5C). CM5 and CM10 swelled up to around 15-20 % of their initial hydrated volume. In contrast, CM20 and CM40 swelled up to 40% and 80% of their initial volume, respectively, evidencing their greater expand upon water uptake.

3.6 Stability against accelerated degradation by collagenase

When put in contact with a collagenase solution, collagen matrices concentrated up to 20 $mg.mL^{-1}$ were fully degraded within a two hour period (Figure 6). The degradation was delayed for CM40 as only 20% of the initial weight was digested after 2 hours. At the end of the experiment (6 h), these matrices were not completely degraded with 20% of the initial weight remaining in a gel form.

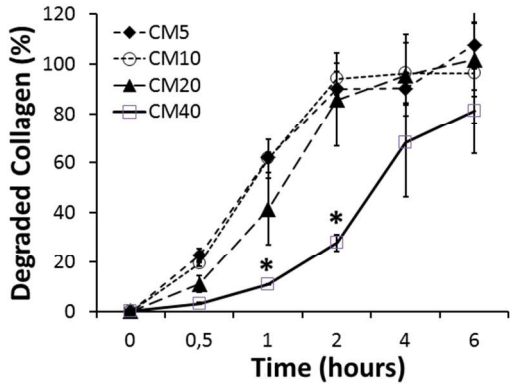

Figure 6: Resistance against accelerated degradation by collagenase. (*: $p<0.05$, One way Anova, post-test: Tukey).

3.7. Drug loading capacity of dense collagen matrices

Since the CM40 materials combine optimal mechanical and biological stability, they were selected for further investigations of their drug release properties. After impregnation of freeze-dried matrices with ampicillin solutions, the loading yield was *ca*. 50 wt % for all antibiotic concentrations (Table 2).

Table2: Loading of CM40 with ampicillin

	CM40Ampi2	CM40Ampi20	CM40Ampi200
Loading (mg/matrix)	1.0 ± 0.1	11.2 ± 4.0	104.1 ± 21.0

However, the incubation of CM40 in a 200 $mg.mL^{-1}$ ampicillin solution revealed to impair its structure (Supporting information 5). These CM40Ampi200 were softer and more transparent than CM40 matrices with lower ampicillin content. In addition, they were difficult to handle. Interestingly, when CM40Ampi200 were freeze-dried and rehydrated in PBS, they recovered their original macroscopic aspect (Supporting information 5).

SEM and TEM imaging indicated that the fibrillar structure of the collagen network was not modified in CM40Ampi2, CM40Ampi20 after rehydration (Figure 7). Matrices were characterized by a collagen network of cross-striated fibrils in which bundles were visible. In contrast, these bundles had disappeared in CM40Ampi200 (Figure 7).

The resistance against enzymatic degradation revealed a poor stability of CM40Ampi200 (Supporting information 6). Their digestion by collagenase was accelerated to reach around 50% of the initial weight after 2 hours. In contrast CM40Ampi2 and CM40Ampi20 exhibited the same behaviour of degradation than CM40 (c.a 30% after 2 hours).

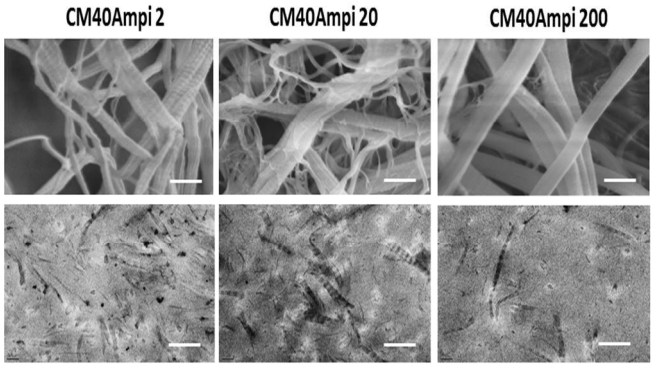

Figure 7: Ultrastructure of CM40 after ampicillin loading. Observation by scanning (top images) and transmission (bottom images) electronic microscopy. S.E.M: Bar: 100 nm. T.E.M : Bar : 200 nm.

3.8 Antibacterial activity and drug release kinetics

CM40s loaded with different ampicillin quantity were evaluated as inhibitors of the bacterial growth of *Staphylococcus Aureus.* For CM40Ampi200, the antibacterial effect was observed after one day and continued over 4 days when matrices were removed and placed into new agar plates on a daily basis (Figure 8).

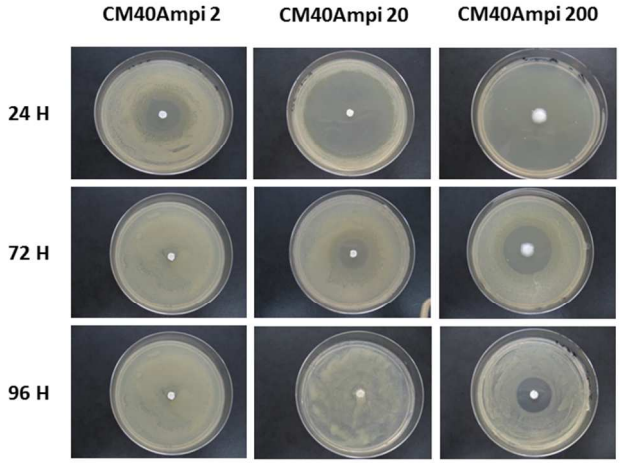

Figure 8: Inhibition of bacterial growth by ampicillin loaded CM40

The antibacterial effect was shorter with CM40Ampi2 and CM40Ampi20 as an inhibition zone was visible over 1 and 3 days, respectively. Quantification of the ampicillin release over 24 h was performed each day using the standard curve and the cumulative released dose was calculated for each matrix and presented Table 3. The minimal inhibitory concentration (MIC) of ampicillin for *S. Aureus,* determined by a liquid-phase method, was 250 $ng.mL^{-1}$ (Supporting information 7). As the matrix volume is 100 µL, if the released quantity of ampicillin is more than 25 ng per day, it means the concentration inside the matrices was higher than the MIC. From the results in Table 3 and Figure 8, each time an inhibition zone was observed, the ampicillin concentration within the matrices was higher than MIC.

Table3: Released quantity (µg) of ampicillin from loaded CM40

	24 H	48 H	72 H	96H
CM40Ampi2	2.01	0	0	0
CM40Ampi20	165.33	1.34	0.50	0
CM40Ampi200	783.24	98.44	2.38	0.07

3.9 Cytotoxicity of the ampicillin-loaded matrices

Cytotoxicity of collagen matrices towards surface-cultured primary fibroblasts was evaluated by Alamar Blue assay after one and six days (Figure. 9). Results were expressed as a percentage of viability assessed in control samples (without CM40) at day 1. Fibroblasts exhibited a high viability after 24 hours, independently of the presence of ampicillin. In addition, the antibiotic release did not impact on cell proliferation as the fibroblasts population was multiplied by ca. 1.5 regardless of the ampicillin concentration. This evidenced the absence of cytotoxicity of the CM40 matrices as such or after ampicillin loading.

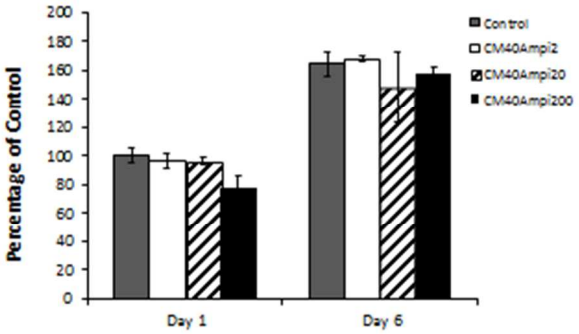

Figure 9: Cellular toxicity of ampicillin-loaded CM40.

4. Discussion

The aim of the present study was to assess the performance of dense collagen matrices as medicated wound dressings. Physical properties, structure, *in vitro* and *in vivo* stability were analysed with regards to the collagen concentration. Thereafter, ampicillin release from the densest matrices was evaluated over four days.

4.1 Structure and mechanical properties of dense collagen matrices

In a previous study, concentrated collagen hydrogels were obtained by modification of the Bell's method [18]. Using concentrated collagen solutions, hydrogels up to 5 mg.mL^{-1} were obtained by neutralization of collagen solutions with NaOH [23-25]. However, highly concentrated hydrogels (more than 5 mg.mL^{-1}) were impossible to synthesize due to the gelling process. Indeed, NaOH is difficult to mix homogeneously with viscous concentrated collagen solutions. In order to fabricate hydrogels with a higher content of collagen, called dense collagen matrices, we performed the gelling of highly concentrated solutions (from 5 to 40 mg.mL^{-1}) under ammonia vapours as previously described [20-22]. The diffusion of NH_3 gas within viscous solutions permitted the generation of dense collagen matrices without dilution.

Compared with hydrogels concentrated at 5 mg.mL^{-1} formed by NaOH addition, the elastic modulus presently measured for CM5 was three times higher [24-25]. This mechanical behaviour can be explained by the presence of thicker fibrils in CM5 since a relationship between fibril diameter and mechanical properties has already been established in the literature [26-27]. Hence, the process of fabrication based on gelling under ammonia vapours has improved the matrix mechanical properties

In these conditions, collagen matrices concentrated at 5 and 10 mg.mL^{-1} exhibited similar structures under electronic microscopy imaging. The only noticeably difference was a slightly higher density of fibrils in CM10 compared to CM5. As explained above, such a structural similarity can explain their close mechanical behaviour. Therefore the benefit of doubling collagen concentration is not obvious from the mechanical properties point of view.

When the collagen concentration increased, diameter and length of fibrils sharply rose. In addition, fibrils bundles appeared in CM40. As a result, the porosity was greater in these matrices. Several authors have shown that the fibril formation was initiated in solution during the lag phase of gelling. Some cross-striated protofibrils with a 5 nm diameter are generated that then assemble in mature fibrils by lateral fusion [28]. The presence of larger fibrils could be explained by a higher protofibrils concentration in CM40. Gobeaux and coll. observed that the fibril diameter increased as the collagen concentration rose within the concentration range presently studied [29]. Our results are in accordance with these results and explain the better mechanical properties measured in CM40. Indeed, stiffness depends on collagen fibrils diameter, and this depends on collagen concentration [26, 30].

4.2 In vivo stability and biocompatibility

The analysis performed after *in vivo* implantation in subcutaneous pockets showed that CM5s were fully colonized after 15 days. In addition, a large part of these matrices was remodelled or digested. In a context of cutaneous chronic wounds, CM5s are not stable enough to be used as a wound dressing because of their large porosity observed by SEM. Chronic wounds are filled by inflammatory cells which produce metalloproteinases [31]. Hence, these cells could rapidly colonize the matrices and digest them. In contrast, CM40s appeared to be stable materials. First, materials retained their original shape and volume. Second, the histological analysis revealed that CM40s were not colonized by cells after 15 days post-surgery. Nevertheless, unlike synthetic hydrogels which are not colonized by cells, CM40 are infiltrated by host cells after one month post-surgery[32]. No inflammatory cells such as macrophages or neutrophils were detected within or around the implants. Last, no large fibrous cap was visible at this time point. This evidences the high tolerance and stability of these materials. Importantly, these results differ from the study performed by Badylak and

coll. claiming that chronic inflammation might take place if cells do not rapidly infiltrate biomaterials [33] .

4.3 Swelling properties and resistance against enzymatic degradation

The swelling ability of CM5 and CM10 was very low. After 11 days, they recover less than 20% of their original hydrated form. Furthermore, freeze-dried CM5 and CM10 underwent contraction just after adding PBS. This is in agreement with previous reports indicated that that low concentrated collagen matrices behave like sponges [34-35]. In terms of applications, it means that CM5 and CM10 are not appropriated to absorb large amount of exudate and would therefore constitute poor wound dressings.

In contrast CM40s behaved like gels during the swelling process [34]. CM40s did not retract and swelled to reach 80% of their original volume at day 4. Used as dried materials, CM40s would be able to absorb a large amount of exudates when applied to a skin wound. In addition, CM40s can be stored in their dried form which represents an advantage for an industrial development. Finally, it is important to point out that CM40 possesses a capacity for liquid absorption similar to products fabricated from alginate, which are currently used as wound dressings [36].

Another important property of these matrices is their delayed degradation. Considering wound dressing applications, two options can be considered: stable materials that have to be removed from the wound after healing (or replaced after some time) or biodegradable materials. In the first case, wound dressings composed of synthetic polymer or natural hydrocolloids such as carboxycellulose or alginate appear the most suitable due to the absence of corresponding proteolytic enzymes in mammalian cells [37-38] . In the second case, an optimal stability has to be defined which relates to the time required for tissue regeneration. Here, we evidenced that the degradation by collagenase was delayed in CM40 compared to loose collagen matrices. This higher resistance is probably due to the fibrils density and thickness within these matrices, as enzymes take more time to digest thicker fibrils.

CM40 may act as a lure for proteases present in the wound bed, protecting the tissues from degradation [31, 39]. This is advantageous compared to alginate-based dressings that are not biodegradable. However, these dense matrices exhibit a lower resistance towards degradation compared to cross-linked collagen sponges. It was previously shown that only a fraction of those materials are degraded after several days and the enzymatic digestion is related to the degree of reticulation [40-41]. To overcome this problem, we attempted to use glutaraldehyde as a cross-linker for CM40. Matrices became brittle and barely handled, suggesting that this option should not be further considered (data not shown). Alternatively, incorporation of drugs that favour wound closure should allow the decrease of the required stability period of the dressing [6].

4.3 Ampicillin release from dense collagen matrices

In this perspective, we focused here on the prevention of infection of cutaneous chronic wounds. Indeed biofilm formation may lead to amputation [8]. In this context, the prolonged delivery of antibiotics within the wound bed is an appropriate strategy. In this study, ampicillin was chosen as molecule model because it belongs to the family of beta-lactam antibiotics. Ampicillin has a broad spectrum against bacteria infecting cutaneous wounds such as *Staphylococcus Aureus* and is easily detected by UV spectrophotometry. To load CM40 with ampicillin, dried materials were incubated with different concentration of antibiotics. Matrices could be loaded up to about 100 mg per matrix. Unfortunately, at this concentration, the materials lost their structure and became sticky and slimy.. This alteration was not due to a pH decrease but probably to an increase of osmotic pressure or ionic strength. This hypothesis is supported by the outcome of the drug release experiment where the osmotic pressure was at its lowest level and the matrices recovered their initial aspect. However, CM40 loaded with 100 mg of ampicillin does not seem to be appropriate for an application as wound dressing because of its poor stability in a proteolytic environment. Indeed, the matrix resistance against the enzymatic degradation by collagenase revealed a rapid digestion of these materials.

In contrast, CM40 loaded with about 11 mg of antibiotics did not shown any structural modifications and allowed an effective release over three days. Moreover, using a standard curve, the ampicillin concentration measured within CM40 was higher than the Minimal Inhibitory Concentration during this period. Measuring the drug release on a petri dish mimicked the *in vivo* release kinetic because wound dressing are applied onto wounds, *i.e* the liquid diffusion comes from the bottom part. As a comparison, collagen sponges previously evaluated as drug delivery system led to full antibiotics release within a 16 hours period [42]. This explains why collagen sponges loaded with antibiotics are not used to prevent infection in opened wounds. Their utilization is restricted to treatment of closed wound after a cardiac or an intestine surgery [43]. In contrast, the here-described dense collagen matrices combine a slow yet effective biodegradation with a sustained antibiotic release. Indeed it is now necessary to identify optimal conditions to achieve a better coincidence between degradation rates and drug release kinetics in order to insure full closure of the wound before complete disappearance of the dressing.

5. Conclusions

We have demonstrated that dense collagen matrices can be considered as promising materials for the development of medicated wound dressings. Their mechanical properties are fully adapted to handling and suturing, they exhibit suitable cyto- and bio-compatibility. They demonstrate resistance against degradation both *in vitro* In addition, they exhibit good swelling properties allowing for the absorption of wound fluids and preservation of moisture in the wound bed. Finally they can be loaded with a large quantity of antibiotics that are released over several days. Taken together, these results give promise for the treatment of cutaneous chronic

wounds. However, further in vivo investigations should be performed in an impaired wound healing model to confirm these results.

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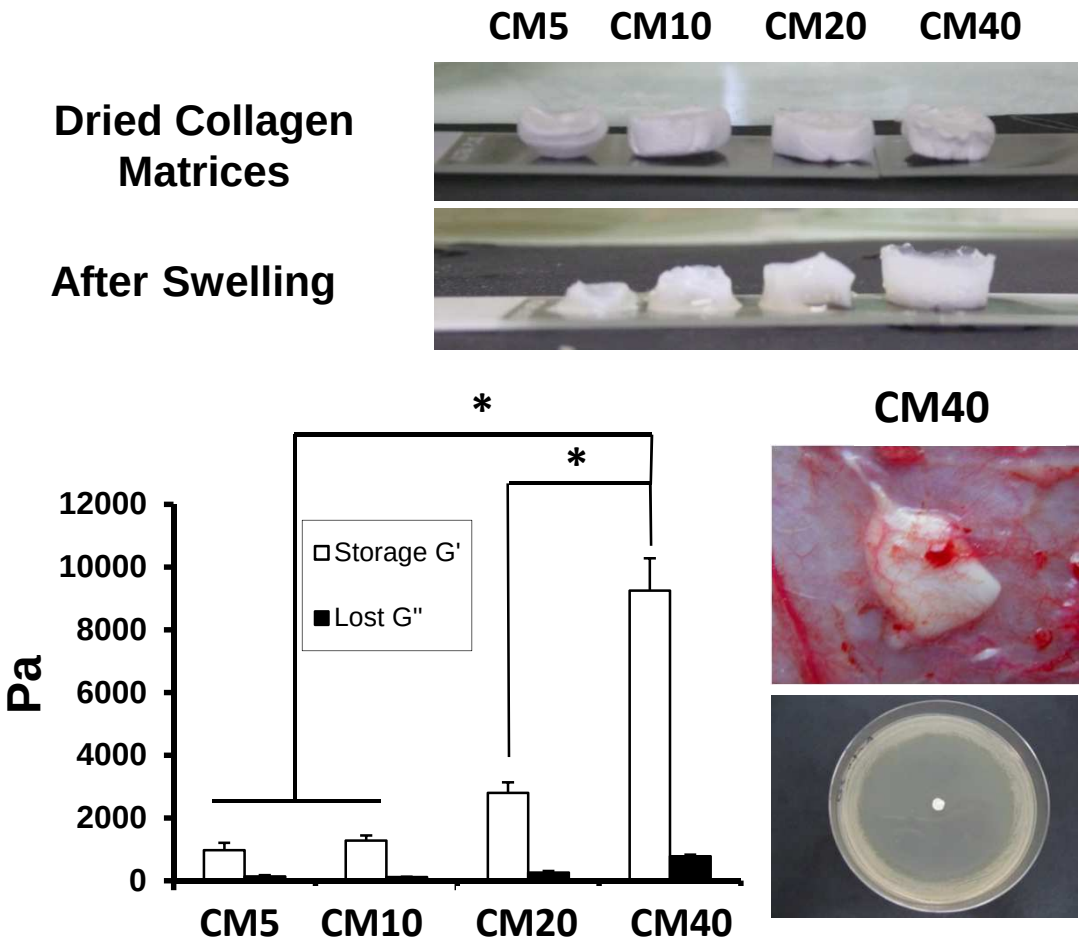

Dense collagen matrices possess high physical properties and allows for the antobiotic release over three days. These biomaterials are promising for an application as medicated wound dressing.