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ORIGINAL ARTICLE

CLOSTRIDIAL COLLAGENASE IMMOBILIZED ON CHITOSAN NANOFIBERS FOR BURN HEALING

Marcela Slovakova [1]✉, Renata Köhlerova [2], Petra Dvorakova [1], Veronika Vanova [1], Martina Spackova [3], Marcela Munzarova [4]

[1] Faculty of Chemical Technology, University of Pardubice, Studentska 573, 532 19 Pardubice, Czech Republic
[2] Department of Medical Biochemistry, Faculty of Medicine in Hradec Kralove, Charles University, Simkova 870, 500 03, Hradec Kralove, Czech Republic
[3] Elmarco s.r.o., Svarovska 621, 460 01 Liberec, Czech Republic
[4] Nano Medical s.r.o., Amperova 650, 463 12 Liberec, Czech Republic

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Summary

This article describes the characterization and application of collagenase-based chitosan nanofiber membranes with rat burns. Electrospun chitosan nanofibers were functionalized with clostridial collagenase using carbodiimide chemistry. The immobilized collagenase was characterized by enzyme activity, kinetic constants, and dry storage stability measurements using a Pz-peptide substrate. The apparent kinetic constants K_M and V_{max} of immobilized collagenase showed a high affinity for the peptide substrate compared to the free enzyme. Drying of chitosan membranes with immobilized collagenase ensured 98 % stability of enzyme activity after rehydration. The effect of collagenase immobilized on chitosan nanofibers on the burn of the rat model was compared with a control treatment with chitosan nanofibers. The healing of the wound with both materials was terminated after 30 days at the same time, although the collagenase wound healed more rapidly during healing. The scar area size after the application of collagenase-containing chitosan nanofiber membranes was 31.6 % smaller than when only chitosan nanofibers were used.

Key words: wound healing; immobilization; collagenase; chitosan; nanofibers

Introduction

Collagenases perform collagen catabolism in various fields of medicine and medical research (1). Collagenases from *Clostridium histolyticum collagenases* are matrix metalloproteases (MMPs) that attack multiple sites along the collagen helix (2). Due to their unique activity in collagen fibers, *C. histolyticum collagenases* have found wide application in the isolation of specific cell types from the accompanying connective tissue, while cells remain intact (1, 3). Collagenase-based applications are known as therapeutic agents for the treatment of collagen-related conditions, such as chronic wounds, burns, ulcers, Dupuytren's contracture, etc., and various scar disorders in various fields of medicine (1, 4). The use of clostridial collagenase that degrades various types of collagen and gelatin is the essence of enzymatic wound cleaning, a commonly used technique to remove necrotic tissue from a wound (5-8).

✉ University of Pardubice, Faculty of Chemical Technology, Studentska 573, 532 19 Pardubice, Czech Republic
marcela.slovakova@upce.cz

Wound healing is a natural but complex, dynamic, and complex process that involves successive phases with a large number of cells, cytokines, growth, and regulatory factors. Recently, the mechanism of expression of human MMP1 was exposed in injured keratinocytes (9). Regenerative medicine is based on the renewal and regeneration of damaged cells, tissues, or organs in a natural way (1). Debridement therapy using enzymes reduces the rate of infection, removes barriers, such as necrotic tissue, to wound healing, and provides an optimal environment for healing (10-15). Microbial collagenase has been confirmed to be a safe and effective choice for the removal of skin ulcers and pressure ulcers (7, 16, 17) and burns (13, 18-20) or in combination with local antimicrobials (21, 22). The use of products containing microbial collagenase is known in the treatment of tissue debridement, in which the support of the granulation and epithelialization processes has been confirmed in damaged, bacterial-infected, or burnt skin (23), and collagenase ointment contributes to the resolution of persistent wound inflammation (5). In several cases, collagenase ointment has proven more effective than, for example, hydrocolloid dressing (17) and the use of medicinal honey (16).

The labile nature of collagenase activity is addressed by repeated application of a dose of collagenase ointment. Enzymes in the form of ointments are covered with a sterile bandage after application (24). Another solution is to immobilize or fix the enzyme on a cover or carrier film or membrane (25). This, in addition to fixing enzyme activity and improving storage stability (26), also has the ability to provide controlled enzymatic debridement (27). For different applications, enzymes were attached to the structure of the material by physical adsorption, ionic, or covalent bonds. The strongest bond is the covalent bond, which prevents the release of the enzyme from the carrier and maintains its high activity. Increased enzyme activity after immobilization and prolonged enzyme stability are key positive properties when both a suitable immobilization method and a material with other properties were used. Enzymes fixed in materials or solid supports have important medical applications. An example is immobilized lysozyme in chitosan-EDTA/PVA nanofibers, which showed a faster reduction in wound size during wound healing (28). Nanomaterials produced by electrospinning polymers in combination with other drugs are currently a well-studied field. Porous nanofiber membranes, which are easily produced by electrospinning, offer a promising solution for wound management. That is, the structure of the matrix, the functionality of the surface, and the rate of degradation of the matrix (20, 29). The formulation of collagenase nanomaterials for meniscus healing confirmed partial digestion of the wound interface and improved repair by creating a more flexible and porous microenvironment that accelerates cell migration and / or proliferation at the wound edge (30). In another example, collagenase was tested on PCL nanofibers with TiO_2 in cells as a scaffold for tissue engineering applications that mimic tissue properties (31). Collagenase-associated nanoparticles have shown the ability to degrade proteolytic tissue and move through the in vitro extracellular matrix (32, 33). The chitosan hydrogel in combination with the hydrating and antimicrobial effects of chitosan had advantageous properties in skin tissue regeneration (34). Chitosan is a universal natural long-chain biomaterial resulting from the deacetylation of chitin, one of the most abundant natural polysaccharides found in the cell wall of microorganisms such as yeast or other fungi in the exoskeletons of crustaceans and insects. The nanofiber form of CS provides other useful properties, such as a considerable specific surface area and high porosity, leading to good permeability to oxygen and water (35, 36). These properties promote cell respiration, skin regeneration, moisture retention, removal of excretion, and hemostasis.

The aim of this work was to contribute to our knowledge of the immobilized enzymatic activity of clostridial collagenase in nanofibrous chitosan membranes and its therapeutic effect on burns on rat skin. The prerequisite was the cleansing effect of the proteolytic enzyme and the antimicrobial effects of chitosan in nanofibrous form. Thus, collagenase immobilized on chitosan nanofibers could form a suitable combination for the use of a covering material in the enzymatic debridement of a burn.

Materials and Methods

1. Chemicals, Materials, and Animals

Collagenase NB 4G from *Clostridium histolyticum* (Collagenase A, EC 3.4.24.3) (70 - 120 kDa) contains class I and class II collagenase and low endotoxin content, Pz activity due to Wünsch: ≥ 0.18 IU/mg and Pz-peptide (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, Mr = 776.9) were purchased from Serva Electrophoresis GmbH (Germany). Samples of chitosan nanofibers and those laminated to gelatine, produced using KiOnutrime-CS

(Kitozyme, Belgium) and polyethylene oxide (Scientific Polymer Products, NY, USA), were prepared by the modified needleless technology NanospiderTM in the NS LAB 500 S electrospinning laboratory device (10 gsm) and were stabilized by heat treatment (130 °C for 1 hour) as described in (37). Zucker Diabetic Fatty Rat (ZDF), healthy controls wild type +/+, originated from the Charles River Laboratories International, Inc. (USA) and purchased from Anlab (Prague, Czech Republic).

2. Immobilization of collagenase on nanofibers

The collagenase enzyme was immobilized in weighed squares of chitosan nanofiber (1.5 × 1.5 cm, 1.65 ± 0.40 mg) according to (38). Nanofibrous squares were hydrated using 1 ml of 0.01 M phosphate buffer (pH 7.3) and after removal of the supernatant, the zero-length crosslinker EDC (7.5 mg) and the sulfo-NHS reagent (1.25 mg) were added. The immediate addition of collagenase (3 mg) dissolved in 0.5 ml of 0.01 M phosphate buffer pH 7.3 was followed and the total volume of 1 ml of the same buffer was ensured. The immobilization proceeded at 4 °C for 16 h with a slight rotation. Collagenase chitosan nanofibers were washed three times using 1 ml of 0.1 M phosphate buffer (pH 7.3), 1 ml of the same buffer containing 1 M NaCl and twice with 1 ml of 0.1 M phosphate buffer (pH 7.3). The same procedure was followed for collagenase sorption on chitosan nanofibers, except for activation by EDC and sulpho-NHS crosslinkers. Squares of collagenase chitosan nanofibers were stored at 4 °C in the same buffer or dried (on polypropylene support) occurred freely in air overnight (24 hours) in Petri dishes at room temperature.

3. Determination of collagenase activity and protein amount

The enzymatic activity of free or immobilized collagenase was estimated by measuring the hydrolysis yield of a standard solution of a freshly dissolute chromogenic substrate Pz-peptide, in 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM CaCl2 at 25 °C according to the optimized conditions described in (38). Detection of the yellow fragment Pz-Pro-Leu was spectrophotometric after extraction with ethyl acetate solution. The experimental values for the functional collagenase molecules per milligram of nanofiber squares were calculated on the basis of a standard curve of collagenase concentrations of known activity. The assay was linear over an enzyme activity range of 0.09 – 1.3 x 10^{-3} IU/ml. The activity of the free and immobilized enzyme was determined in terms of active units (U), where 1 U catalyzes the hydrolysis of 1 μmole 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg per minute at 25 °C, pH 7.1.

The amount of immobilized collagenase was determined using the Micro BCA™ Protein Assay Kit (Thermo Scientific) in terms of protein micrograms per mg (μg/mg) of the nanofiber sample. Subsequently, quantification was calculated from the calibration curve of the collagenase solution in 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM $CaCl_2$.

All measurements were repeated a minimum of two times, the calculated means and SD values of which are shown in the graphs.

4. Determination of kinetic constants

The Michaelis–Menten kinetic parameters K_M and v_{max} for the hydrolytic reactions between the Pz peptide and collagenase were determined from the Lineweaver–Burk plot with 5 different substrate concentrations. The concentration range of the substrate Pz peptide was 0.2 – 0.9 mM. 10 ul collagenase, 100 μl substrate, 140 μl 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM $CaCl_2$.

5. Storage and drying of the collagenase chitosan nanofiber

Collagenase chitosan nanofibers were stored in 1 ml of 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM $CaCl_2$ at 4 °C. Drying (24 h) and storage of dry collagenase chitosan nanofibers occurred in the air at laboratory temperature. Before determining collagenase activity, collagenase chitosan nanofibers were hydrated for 10 min in ultrapure water and then washed with ultrapure water (3x 1 ml) and finally with 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM $CaCl_2$ (1 x 1 ml).

6. Application of nanofiber membranes to burn

To investigate the healing effect of collagenase immobilized chitosan nanofibers, six male ZDF (+/+), 24.7-week-old laboratory animals with an average weight of 377 g were used for the experiment. This study was carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Animal Welfare Committee of Charles University in Prague, Faculty of Medicine in Hradec Králové (MSMT-20364/2013-14). The anesthetized and analgesic subcutaneous skin was shaved, disinfected, and two wounds with a diameter of approximately 1 cm were created 2 cm apart on the back. The burn wound was formed on the back with an aluminum seal that was heated in a 100 °C water bath and applied to the skin for 10 seconds. After injury, the animals were housed separately and analgesics (0.05 mg / kg buprenorphine hydrochloride, IVAX, Opava-Komarov, Czech Republic) were administered. The collagenase-filled nanofiber membrane (approx. 1 cm^2) was moistened with saline prior to application. A control sample of 1 x 1 cm chitosan alone was applied dry on a polypropylene substrate, which was then moistened with saline and applied without the polypropylene substrate. Subsequently, all wounds were covered with a gauze square, which was fixed with plaster, and the body of the rat was bandaged with a bandage and subsequently fixed with an elastic bandage. The wounds were exchanged and bandaged twice a week eight times in a row or until they healed with a new nanofiber sample.

7. Evaluation of wound healing

The wounds were photographed (EOS D350, Canon, Tokyo, Japan) using a millimeter ruler immediately after induction (day 0) and then every third or fourth day. Day 0 wounds were not allowed for maximal retraction before being photographed. The wound area was measured using ImageJ software (NIH) calibrated to a standard length using a millimeter ruler. The relative wound area size was expressed according to the following formula: % wound area is wound area on day n/wound area on day 0. The percentage share of epithelialization in wound repair was equal to % epithelialization scar area/wound area on day 0.

$$\% \text{ wound area} = \frac{\text{wound area on Day n}}{\text{wound area on Day 0}} x\ 100 \qquad (1)$$

$$\% \text{ share of epithelialization} = \frac{\text{epithelialization scar area}}{\text{wound area on Day 0}} x\ 100 \qquad (2)$$

Results and discussion

Immobilized collagenase activity

In this paper, we proposed the immobilization of collagenase on chitosan nanofibers for local application on burns. Chitosan nanofibers were chosen as a suitable carrier for immobilized collagenase, due to their suitable properties for wound healing, such as antimicrobial effects and hydration of coated tissue. The chitosan used for the nanofiber formation was of plant origin, which is safe when used for wound healing. The use of such material for nanofiber formation and use as a biomaterial for wound healing is without risk of transmitting animal diseases.

An immobilization method used water-soluble carbodiimide EDC with the addition of sulfo-NHS. Carboxyl groups of collagenase were activated and reacted with the amine functions of electrospun chitosan. The resulting amide bond is the stable bond that prevents the release of enzyme molecules. The main advantage of the covalent bond over the sorption is the prevention of immobilized collagenase from proteolytic acting on neighboring enzyme molecules. The immobilized collagenase activity was measured against a synthetic peptide substrate (Pz-peptide). The optimal amount of collagenase was tested for immobilization, to find the maximal stable proteolytic activity in the immobilization reactions of 0.05, 0.1, 0.5, 1 and 3 mg collagenase per square of chitosan nanofiber (1.65 ± 0.40 mg). Simultaneously with covalent binding, physical sorption of collagenase was performed to distinguish non-covalent bonds. It can be seen in Figure 1 that the higher the amount of immobilized collagenase, the higher the specific collagenase activity per square of nanofiber was achieved, as well as the amount of protein detected.

Significant differences were observed between each subsequent point. The immobilized collagenase activity had the expected result, the immobilization had an increasing tendency leading to activity from 0.152 IU / mg to 0.960 IU / mg nanofibers. The amount of protein at the lowest collagenase concentration of 0.05 mg resulted in a detected value of 45.6 (plus or minus 0.0015) micrograms, the highest concentration of 3 mg resulted in a detected value of 346 (plus or minus 0.034) micrograms. The trends of increasing enzyme activity and the amount of protein detected indicate a large capacity of the chitosan nanofiber, which begins to decrease slightly at the last concentration. Sorption experiments achieved significantly lower values of the detected amount of protein, in addition to a large standard deviation. They indicate a large proteolytic degradation of the enzyme in a sorption experiment. Specific enzyme activity in the case of adsorbed collagenase were not measured.

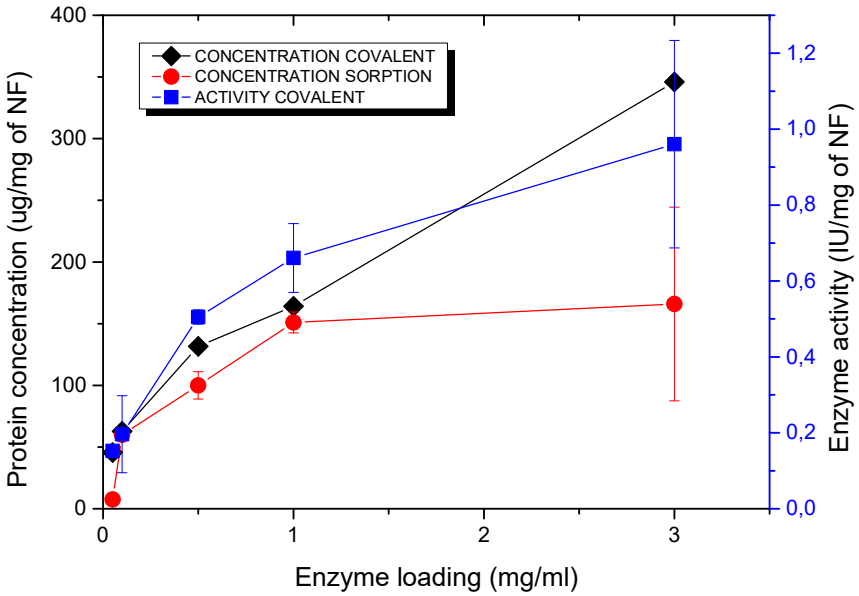

Figure 1. Specific collagenase activity and concentration per milligram of collagenase immobilized or sorbed on chitosan nanofibers. Data are mean values (n=2).

Stability of enzyme activity of dried collagenase chitosan nanofibers

Previously, we observed an unchanged storage stability of immobilized collagenase on chitosan nanofibers in a storage solution for 4 weeks at 4 °C (38). However, the purpose of immobilizing collagenase on chitosan membranes for the healing of burn wounds did not allow long-term storage in solution. For later applications, the collagenase membranes were dried. To ensure sufficient activity of the immobilized enzyme after drying and storage, we performed several comparative experiments with subsequent detection of enzyme activity and characterization of the appearance of membranes by SEM analysis (Figure 2). The squares of nanofibers were dried at room temperature in circulating air, in a fume hood, or loosely for 24 hours until completely dry. Enzyme storage stability had a slightly increased value due to diffusion constraints that reduced the initial observed activity (Figure 2C), also observed by other authors (39). The drying of the nanofiber membrane with immobilized collagenase prior to storage occurred under conditions of 24 hours in free or circulating air (in a fume hood). We compared the resulting enzyme activity with the activity of freshly immobilized collagenase after rapid rehydration of the material. The results in Figure 2C show that drying for 24 hours retains the enzymatic activity of the membrane at 98 % and 96 % of the original activity when dried freely and in circulation, respectively. When the drying process itself was extended to 48 hours, a reduction in collagenase activity was observed to be 47 %. Thus, prolonging the drying time has a significant effect on reducing enzyme activity. Another reason for choosing free drying of the prepared nanofibers with collagenase was the fact that the nanofiber membrane twists more and even folds when dried in forced circulation. Chitosan swells after being soaked in an aqueous solution; however, the structure is largely preserved.

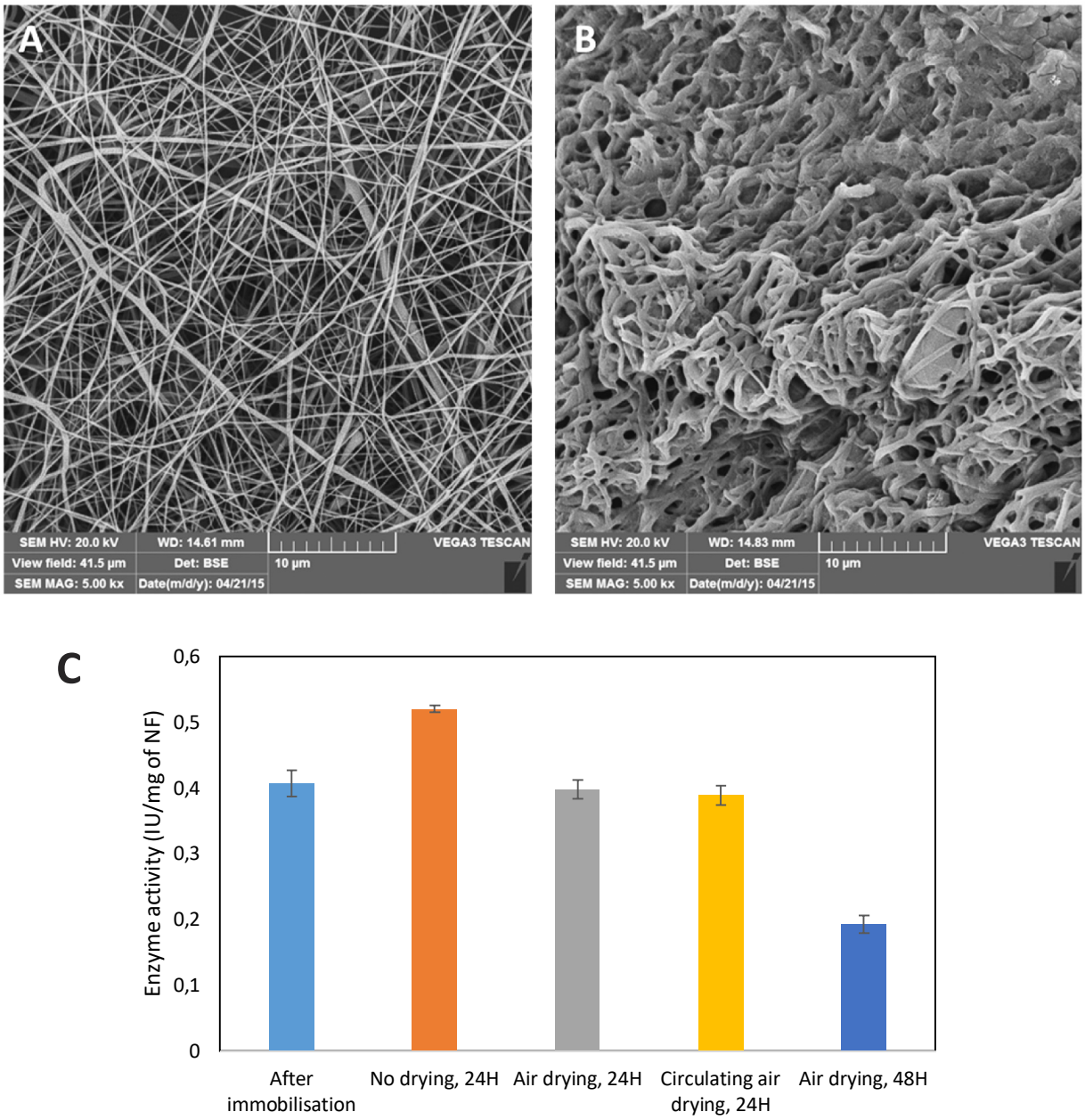

Figure 2. Representative SEM images of the original chitosan nanofiber structure (A) and (B) after hydration and air drying. Scanning electron microscopy of nanofiber samples was performed using a VEGA3 SBU apparatus and a backscattered electron detector (Tescan, Czech Republic). The samples were coated with a conductive layer of gold (thickness 0.2 nm) in a Balzers sputter coater to prevent charging of the sample. Magnification 5,000 x, 10 kV. Scale bars are 10 µm in both SEM micrographs. (C) Specific enzyme activity of collagenase immobilized on chitosan nanofibers under different drying conditions compared to the value of enzyme activity after enzyme immobilization. Data are mean values (n=2). Foto - Ing. Radovan Metelka, Ph.D. University Pardubice.

Kinetic constants of immobilized collagenase

To characterize clostridial collagenase immobilized on chitosan nanofiber biochemically, we measured its affinity to a synthetic peptide substrate based on the collagen sequence, Pz-peptide. The collagenase used in this work is a mixture of class I and II collagenases, but there are differences in the affinity for substrates for each of them.

Class I Clostridium histolyticum collagenases (ColG - α, β, and γ-collagenases) have higher activities against collagen and gelatine and lower activities against synthetic peptides FALGPP, FALGPA, and Pz peptide. Class II collagenase (ColH - δ ∈ and ζ-collagenases) have approximately one-third of the activity against collagen and gelatine, but it has significantly higher activities against synthetic peptides (40, 41). Kinetic constants indicate the rate of enzyme reaction as a function of substrate concentration. The rate constants of the immobilized and soluble enzymes usually differ, including the effect of the material and the immobilization technique. The dependence of the substrate concentration on the reaction rate is described by the Michaelis-Menten equation, and from its graphical representation the Michaelis constant K_M and the maximum reaction rate V_{max} are obtained. K_M expresses the degree of affinity of the enzyme for the substrate; V_{max} corresponds to the state where there is no free enzyme in the reaction (42). If the K_M is high, a high substrate concentration is required, and the affinity is low. KM values are usually around 10^{-2} to 10^{-6} mol/l. We measured and calculated K_M and V_{max} for immobilized and free collagenase (Table 1). Other authors previously characterised the kinetic constants of free clostridial collagenases; to our knowledge, no other authors analyzed immobilized collagenase (2, 43-45). The K_M and V_{max} values revealed a higher enzyme-to-substrate affinity of the collagenase immobilized on the chitosan nanofiber compared to free collagenase. This phenomenon we have already described (46) of immobilized enzymes. An explanation is the increase in the local substrate concentration near the immobilized collagenase molecules. The K_M obtained for immobilized collagenase is an apparent constant. Evaluation of the Michaelis-Menten kinetic constants for immobilized collagenase confirmed that the immobilization technique is suitable for the enzyme and the carrier.

Table 1. Measured kinetic constants of *Clostridium histolyticum* collagenase.

Collagenase type	Class	Form	Substrate	Kinetic constants
Collagenase	I, II	soluble	Pz-peptide	K_M 0.504 mmol/l V_{max} 0.21 mol/l.s
Collagenase chitosan nanofiber	I, II	Immobilized	Pz-peptide	K_M 0.356 mmol/l V_{max} 0.09 mol/l.s

Application of collagenase chitosan nanofibers in a burn wound

Chitosan nanofiber squares with a proteolytic system were previously demonstrated for *in vivo* safety tests of skin irritation and sensitization in the study of cytotoxicity without skin reaction (37). Furthermore, in this study the effect of the reported properties of chitosan as a hydrating and antimicrobial agent is studied in association with collagenase. Clostridial collagenases are capable of breaking down various types of collagen and gelatine, which is the essence of enzymatic wound cleansing. Proteases are also produced *in vivo* to prevent the development of infection. During scar formation, proper remodelling of collagen fibres depends on the balance between their synthesis and degradation. Physiologically, the degradation of collagen fibres is ensured by metalloproteinases, which are produced by neutrophils, macrophages, and fibroblasts (19). Clostridial collagenase immobilized on chitosan nanofiber squares was stored dry at room temperature before wound application. To monitor wound healing by immobilized collagenase on chitosan nanofibers, we used healthy controls of the Zucker diabetic fatty rat, which is one of the animal models used to investigate obesity and insulin resistance. Chitosan nanofiber squares were moistened with saline. In a pilot study, an application of the chitosan sample was compared to the saline under the same conditions. At the end of the experiment, the final wound area was 0% for chitosan and 70% for control saline (data not shown). From these results, a two-wound experiment was designed, in which the effects of samples of chitosan nanofibers and chitosan nanofibers with collagenase were compared to reduce the number of painful and poorly healing wounds.

Two burn wounds with a diameter of 1 cm and approximately 2 cm apart were created on the back of laboratory animals with a heated aluminum seal. One wound was covered with a square of collagenase-immobilized chitosan nanofibers; the other wound was covered with only one square of chitosan nanofiber (Figure 3). For each wound, the area size of the wound was evaluated at regular intervals and, at the same time, the degree of epithelialization, that is, wound healing, was monitored and measured (Figure 4). According to the protocol, the collagenase chitosan

nanofibers were replaced with each wound treatment. According to the protocol, it was planned 8 times, but here it was 6 times. The scab was not removed intentionally, but was sometimes torn off with the bandage to which it stuck. The scab did not appear until the thirteen day after the injury. For the first days, the wounds with both materials did not change and then darkened to form a scaly formation. Upon removal of the scab, it was found that part of the wound is epithelialized and a new scab-like formation is formed on the other part. Wound healing was terminated on the 30th day after initiation a total of six nanofiber exchanges. Figure 3 shows *photographs of the appearance of the wound during healing. It can be seen congestion on the wound place.*

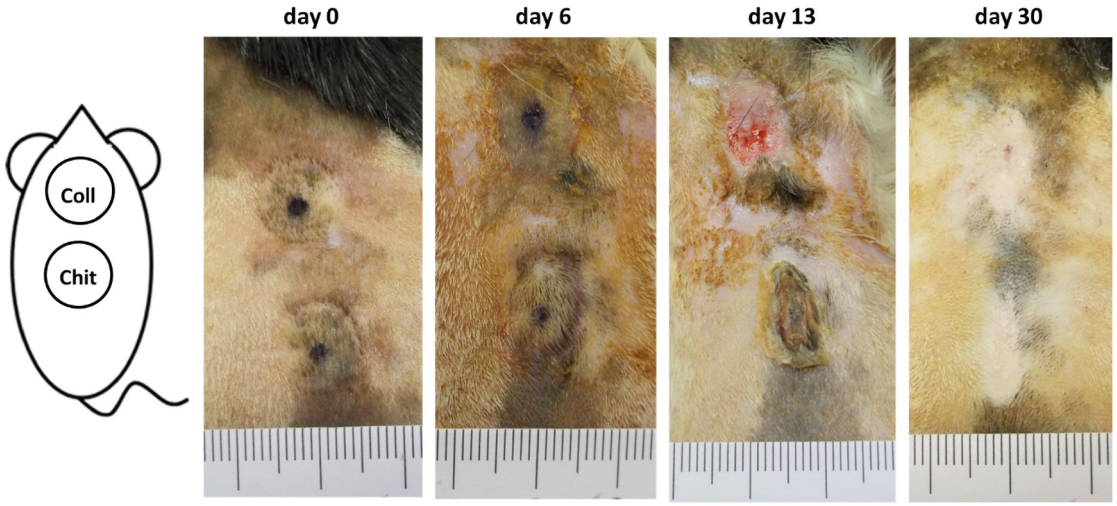

Figure 3. Photographs of skin wounds and subsequent wound contraction on days 6, 13, and 30 after treatment with immobilized collagenase chitosan nanofibers (Coll) and controll chitosan nanofibers (Chit). Scale bar: 1 cm. Foto - Mgr. Renata Köhlerová, Ph.D., Faculty of Medicine in Hradec Kralove, Charles University.

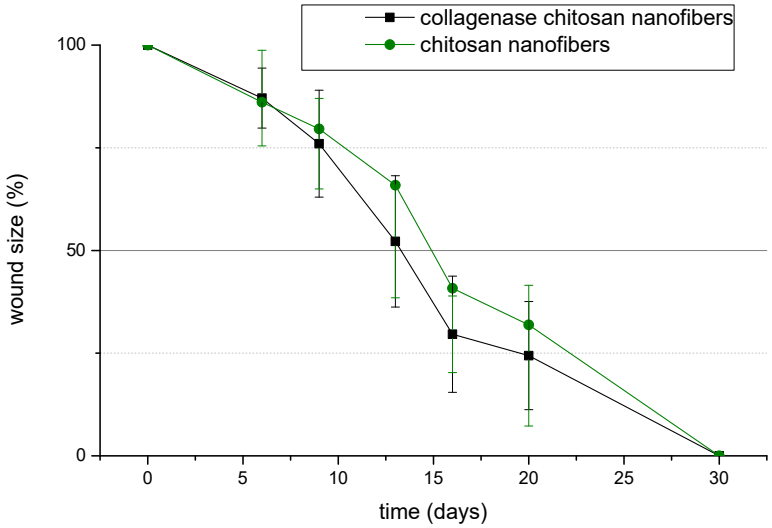

Figure 4. The percentage of the area size of the original wound during healing. Data are mean values (n = 6).

Figure 4 summarizes the results of the assessment of the wound area size, the percentage of the original wound area size. The healing process of the wound treated with collagenase and chitosan was the same until the ninth day after the injury. The collagenase wound then healed more rapidly; the promoter effect of collagenase on the wound

is supposed to be due to its enzyme debridement ability. This includes primarily the degradation of necrotic tissue from a wound. Charernsriwilaiwat et al. observed the same effect in 2012 in the first 1 to 5 days of healing using immobilized lysozyme in chitosan-EDTA/PVA nanofibers (28). Final wound healing occurred in both groups on the same day 30.

We also compared the scar area size with the burn area size on day 30 of the experiment for collagenase immobilized to chitosan nanofibers and the control chitosan nanofibers itself. Scars with both types of healing materials were smaller by 48.3 and 70.6 % at full healing. The scar after the application of collagenase immobilized on chitosan nanofibers was 31.6 % smaller compared to the scar after the application of the square with chitosan nanofibers. Thus, collagenase immobilized on chitosan nanofibers was found to be active and stable and contributed to faster burn healing compared to control chitosan nanofibers.

Conclusion

The selected type of covalent immobilization of collagenase on the functional groups of the chitosan nanofiber proved the suitability of the conditions for the preparation of an active and stable enzyme carrier. From the results of this pilot study on burn healing, it is clear that the use of chitosan nanofibers with collagenase has a direct positive effect on the healing rate and the area size of the resulting scar. Such an effect may be the subject of a more in-depth study with the extension of histological analyses and their significance for cosmetic purposes.

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Author Contributions

MS planning, experiments and writing, RK experiments and writings, MJ and MM preparation of nanofibers, PD and VV experiments.

Conflict of Interest Statement

The authors state that there are no conflicts of interest regarding the publication of this article.

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Adherence to Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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