

TESTING OF COLLAGENASE COVALENTLY LINKED TO CHITOSAN NANOFIBERS FOR BIOMEDICAL APPLICATIONS

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Abstract

Nanofibers biofunctionalized by enzymes is materials of interest in the biomedical field due to their potential application in wound healing. This work describes the production and characterisation of electrospun chitosan nanofibers biofunctionalized by microbial collagenase. The morphology and microstructure of the chitosan nanofibers prepared by Nanospider technology were examined using a scanning electron microscopy (SEM). Amino groups of chitosan nanofiber were connected with the carboxylic acid groups of collagenase using EDAC and sulfo-NHS methodology. Collagenase enzymatic activity was evaluated by peptidic substrate (Pz-peptide, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg) in order to estimate the ability of this biomaterial to be used as an enzymatic debriding wound dressing. Characterisation of collagenase chitosan nanofibers in term of apparent Michaelis-Menten constants ($K_M(\text{app})$ and v_{max}) measurements revealed higher enzyme to substrate affinity in comparison with soluble form of collagenase. The resulting novel collagenase chitosan nanofibers showed excellent operational stability and long shelf life for at least 6 weeks. Therefore, the novel collagenase chitosan nanofibers are expected to be a potential scaffold for wound healing applications, as e.g. enzymatic debridement.

Keywords: chitosan nanofibers, collagenase, enzyme immobilization, Michaelis-Menten constant

1. INTRODUCTION

Clostridial collagenases are used for a broad spectrum of biotechnological applications; they are suitable for isolation of a broad variety of cell types, especially for fibroblasts, human and rodent hepatocytes, frog oocytes and epithelial cells. Generally, they cleave peptide bonds on the amino side of the glycine residue [1]. Clostridial collagenases are capable of degrading various types of collagen and gelatine, which is the essence of an enzymatic debridement, frequently used technique for removal of necrotic tissue from wounds. Microbial collagenase already used as an active ingredient ointment (Irujol® Mono Ointment) is safe and effective choice for debridement of cutaneous ulcers and burn wounds [2-4]. To render this technology clinically feasible, enzymatic degradation must be conducted in a controlled and targeted manner to localize digestion to the wound site. As suitable alternative, one potential delivery vehicle is nanofibrous scaffolds fabricated via electrospinning. In this well-established process, fibers that are hundreds of nanometers in diameter can be formed and compiled into a non-woven 3-D scaffold [5]. Biopolymers used in biomedicine which are biodegradable are either natural (chitosan, collagen, fibrinogen), synthetic (polylactic acid, polyglycol acid), or copolymers. Better biocompatibility and low immunogenicity is seen in natural biopolymers [6]. Chitosan nanofibers prepared by needle-less electrospinning as an enzyme-releasing scaffold have potential to enhance integrative repair [7]. An example of suitable delivery system is collagenase stored inside electrospun poly(ethylene oxide) nanofibers releasing active molecules upon hydration [5]. To prevent effectively both denaturation and leaching of enzyme molecules is use of covalent linkages [8]. Especially, immobilizing of proteases by covalent bond furthermore decreases autocatalysis and controls proteolysis [9].

In this article, we describe the optimized method of covalent collagenase immobilization to electrospun chitosan nanofibers. Collagenase activity as essential knowledge ensuring its active form was investigated using the hydrolysis of peptidic substrate and optimized for using with nanofiber membrane. Other characteristics as storage and operational stability, Michaelis-Menten constant measurement are described. Desiccation as potential storage of collagenase chitosan nanofibers were investigated as well as sterilization conditions.

2. EXPERIMENTAL

2.1. Chemicals and manufacturing of nanofibers

KiOnutrime-CS (Kitozyme, Belgium), polyethylene oxide (Scientific Polymer Products, NY, USA), collagenase NB 4G from *Clostridium histolyticum* (70 - 120 kDa, contains class I and class II collagenase, PZ activity (Wünsch): ≥ 0.18 U/mg) and Pz-peptid (4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, Mr = 776.9) were purchased from SERVA Electrophoresis GmbH, Germany, other pure chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chitosan nanofibers, described in detail in [7], were prepared using the modified needleless Nanospider™ technology on an NS LAB 500 S electrospinning laboratory device (Elmarco Ltd., Liberec, Czech Republic) [10]. Shortly, chitosan nanofibers were prepared from the polymer solution of chitosan (5 hm.% to 20 hm.%) and polyethylene oxide (1 hm.% to 10 hm.%), in acetic acid by electrospinning methodology and were crosslinked by 130°C heat for 1 hour. Nanofibers thickness was analysed by scanning electron microscopy (SEM) using VEGA3 TESCAN microscope. Chitosan nanofibers of basis weight 20 [g/m²] were cut into squares (1.5 × 1.5 cm). Prior to biofunctionalization of the nanofibers, polypropylene spunbonds were then torn off and all squares were weighted.

2.2. Collagenase immobilization onto chitosan nanofibers

The enzyme collagenase was immobilized on the 1.5 × 1.5 cm chitosan squares according to [11] with slight modifications. Nanofibrous squares were rehydrated by 1 ml of 0.01 M phosphate buffer (pH 7.3). The supernatant was removed and the zero-length crosslinker EDC (7.5 mg) and sulfo-NHS (1.25 mg) reagent (each dissolved in 0.2 ml of 0.01 M phosphate buffer (pH 7.3)) were quickly added to the nanofibers. Immediate addition of collagenase solution followed (unless stated otherwise, 3 mg of collagenase dissolved in 0.5 ml of 0.01 M phosphate buffer (pH 7.3)) and 0.1 ml of the same buffer was added. The immobilization proceeded at 4°C for 16 h under mild rotation. Subsequent washing was carried out by 3 times with 1 ml 0.1 M phosphate buffer (pH 7.3), once with 0.1 M phosphate buffer (pH 7.3) with 1 M NaCl and 2 times with 0.1 M phosphate buffer (pH 7.3). All measurements were repeated a minimum of two times, the calculated means and SD values of which are shown in the graphs herein.

2.3. Determination of soluble and immobilized collagenase activity

The enzymatic activity of the soluble or immobilized collagenase was estimated by measuring the hydrolysis yield of a standard solution of a freshly prepared chromogenic substrate, Pz-peptide, in 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM CaCl₂ according to [12]. 1 U according to Wünsch catalyzes the hydrolysis of 1 μmole 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine per minute at 25 °C, pH 7.1. Resulting 4-phenylazobenzoyloxycarbonyl-Pro-leucin has after its extraction to ethylacetate changed to yellow product which was measured spectrophotometrically. In the detail, activity of the 0.02 ml soluble or immobilized enzyme was determined in terms of micrograms (μg). The hydrolysis of 1.29 mM Pz-peptide was performed in a 0.03 M TRIS-HCl buffer (pH 7.0) containing 0.2 M NaCl and 5 mM CaCl₂ at 37°C (final reaction volume 2 ml). Enzymatic reaction occurred under mild stirring and stopped after 25 min by addition of 0.25 ml 22 wt % citric acid per 2 ml of supernatant. 2 ml of ethylacetate was added, the product was vigorously shaken and the absorbance of the organic phase was measured at 320 nm in quartz cuvette using the UV/VIS spectrophotometer Biochrom LIBRA S22 (Thermo Fisher, CR). Collagenase

activity per mg of nanofibers was then calculated. The activity of soluble collagenase was determined by the same method using the corresponding quantity of immobilized collagenase (4 µg).

2.4. Kinetic measurement

The method of collagenase activity measurement described in 2.3. was used to acquire data for $K_M(\text{app})$ and v_{max} evaluation by standard Lineweaver–Burk plot. For this purpose, a sample of nanofibers (1.5 × 1.5 cm) with immobilized collagenase or corresponding quantity of soluble collagenase (90 µg) was mixed with the Pz-peptide in concentrations of 0.2, 0.3, 0.5, 0.7 and 0.9 mM in 2,250 ml and incubated in 37°C. Aliquots of 0.24 ml volume were then stopped by 1 ml of 3% citric acid, extracted to 2 ml of ethyl acetate. The change in absorbance at 320 nm of organic phase was monitored in 3 min intervals for an overall time of 24 min on a UV/VIS spectrophotometer Biochrom Libra S22 (Thermo Fisher, CR).

2.5. Storage and desiccation of collagenase chitosan nanofibers

Collagenase chitosan nanofibers were stored in 1 ml of 0.03 M TRIS-HCl buffer (pH 7.0) with 0.2 M NaCl and 5 mM CaCl₂ with sodium azide (0.1% w/v) at 4°C. Desiccation (24 h) and storage of desiccated nanofibers occurred in the air at laboratory temperature. Prior to determining collagenase activity, collagenase chitosan nanofibers were rehydrated for 10 min in 2 ml of ultrapure water and then washed with ultrapure water (3 × 1 ml) and finally with 0.03 M TRIS-HCl buffer (pH 7.0) with 0.2 M NaCl and 5 mM CaCl₂ (1 × 1 ml).

2.6. Sterilization of collagenase chitosan nanofibers

Desiccated collagenase chitosan nanofibers were irradiated microbicidal UV-C radiation ($\lambda = 253.7$ nm) or decontaminated with 80% ethanol. First method comprises irradiation from both sides from length 1 m during 30 min. Afterwards; nanofibers were rehydrated in 2 ml of sterile distilled water and washed 3 times in 0.03 M TRIS-HCl buffer (pH 7.0) with 0.2 M NaCl and 5 mM CaCl₂. Decontamination of collagenase chitosan nanofibers included incubation in 80% ethanol for 5 min, in 50% ethanol for 10 min and in sterile distilled water for 10 min at room temperature. Collagenase chitosan nanofibers were then washed 3 times in 1 ml of sterile distilled water and analogously to the first method, nanofibers were washed by 3 times in 0.03 M TRIS-HCl buffer (pH 7.0) with 0.2 M NaCl and 5 mM CaCl₂.

3. RESULTS AND DISCUSSION

3.1. Preparation of collagenase chitosan nanofibers

The structure of the nanofibers was observed on SEM images (Fig. 1A). The fiber diameter ranged between 100 – 200 nm (exceptionally up to 1 µm) for the chitosan nanofibers. To reveal the stability of nanofibers in water, samples were immersed in distilled water for 1 day, subsequently dried and observed by SEM microscopy (Fig. 1B). After immersion, the diameter distribution was not significantly changed and the nanofibrous structure was apparently preserved.

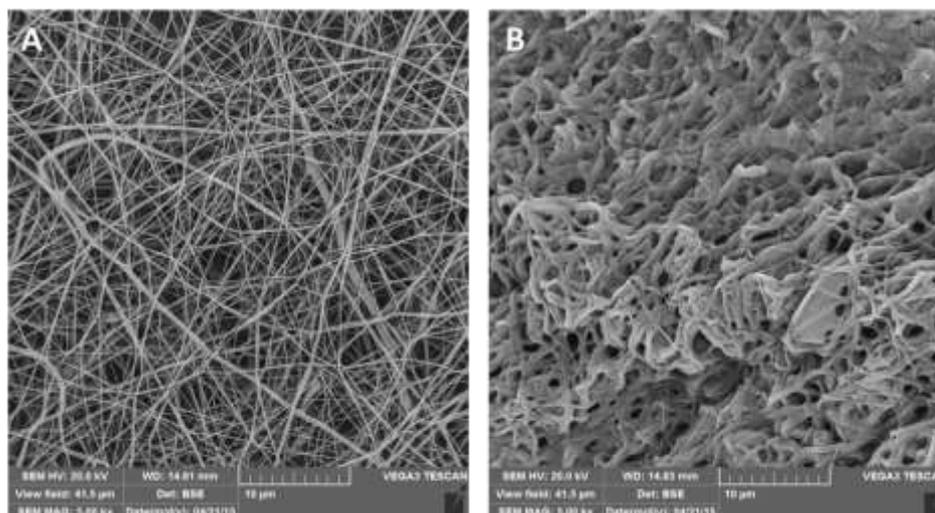


Fig. 1 Scanning electron microscope of chitosan nanofibers before (A) and after (B) hydration, magnification 5,000 x. VEGA3 TESCAN.

The immobilization of microbial collagenase was performed by the EDAC and sulfo-NHS method. The principle of covalent immobilization of collagenase is based upon activation of chitosan nanofiber free carboxylic acid groups by means of 1-(ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDAC). These activated groups of nanofibers together with the 3-sulfo-1-hydroxysuccinimide (s-NHS) that is present provide a reactive ester which is converted into an amide by reaction with the amino groups of collagenase. The digestion efficiency of collagenase chitosan nanofibers was analysed by determining catalytic activity with the specific low-molecular substrate Pz-peptide from two repetitions. Different parameters as amount of EDAC, s-NHS and collagenase, and time of incubation and temperature used in an immobilization process were optimized. Using the most suitable conditions the highest possible level of enzyme activity are shown in the plot of collagenase chitosan nanofibers activity on temperature and time of incubation (Fig. 2). The highest level of collagenase chitosan nanofibers activity (3.51 µg/mg nanofibers) was achieved using 4°C and 7 h incubation. These conditions and surely decreased temperature ensured suitable conditions for low enzyme autolysis and highest enzyme activity yield. Furthermore, resulting immobilized collagenase activity is fully comparable to the ointment Iruxol Mono (0.48-3 mg collagenase / g of ointment) available on the market [13].

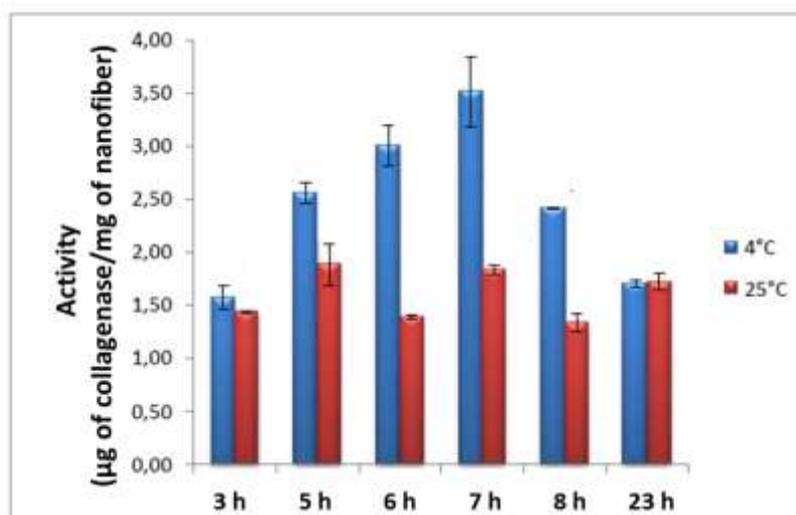


Fig. 2 Use different time of incubation and temperature for immobilization of collagenase to chitosan nanofibers under optimized amounts of chemicals.

3.2. Characterization of collagenase chitosan nanofibers

In this study, we biochemically characterized the affinity of clostridial collagenase NB 4G from *Clostridium histolyticum* to synthetic peptidic substrate Pz-peptide. We undertook Michaelis–Menten kinetic measurement (Table 1). Resulting apparent Michaelis-Menten constant ($K_M(\text{app})$) values are consistent with literature [1], differences causes using different substrates. Kinetic parameters ($K_M(\text{app})$) and v_{max} are important parameters especially in immobilized enzymes because these help to assesses whether the active site steric accessibility (enzyme–substrate affinity) is maintained after enzyme immobilization. The evaluated ($K_M(\text{app})$) for collagenase immobilized on chitosan nanofibers compared to the ($K_M(\text{app})$) for soluble collagenase indicates a higher affinity for the active binding site of collagenase. These lower values correspond to previous observations of enzymes conjugations to material as magnetic particles [14] or polyethylene glycol [15]. A possible interpretation of the lower values against soluble collagenase is that the large specific surface area for hydrophilic nanofibers changes the volume of substrate pre-concentration in proximity to the nanofiber surface.

Table 1 Michaelis constant of soluble and immobilized collagenase.

	Substrate	$K_M(\text{app})$ (mM)	v_{max} (mol/l.s)	References
Collagenase (class I-II) chitosan nanofibers	4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg	0.356	0.09	This work
Soluble collagenase (class I-II)	4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg	0.504	0.21	This work
Soluble collagenase	karbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala	0.71	-	16
Soluble collagenase G (class I)	furylacryloyl-Leu-Gly-Pro-Ala (FALGPA)	0.84	-	1
Soluble collagenase isoform G (class I)		1.26		
Soluble collagenase H (class II)		0.269		
Soluble collagenase isoform H (class II)		0.425		

Additionally, operational and storage stabilities of nanofibers biofunctionalized with collagenase were observed. Due to the operational stability measurement results (Fig. 3), there exists no decrease subsequent for 8 measurements. Storage stability is one of the favoured advantages of enzyme immobilization. To demonstrate long-term usability of immobilized collagenase, the prepared collagenase chitosan nanofibers were stored at 4 °C in storage buffer before the enzyme activity measurement. Results showed no significant decrease in specific enzyme activity (still at 100%) after 6 weeks.

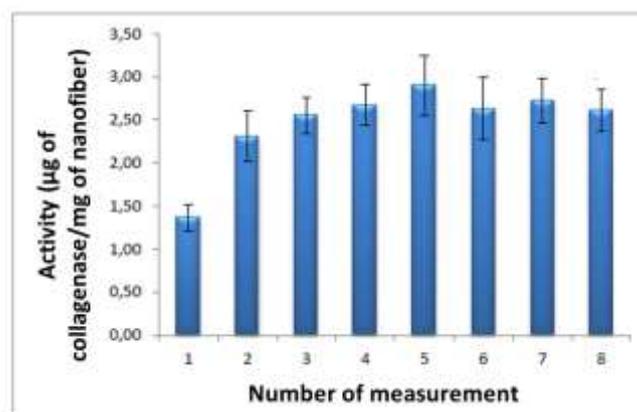


Fig. 3 Operational stability of collagenase chitosan nanofibers.

3.3. Sterilization of collagenase chitosan nanofibers

We further investigated the sterilization conditions of collagenase chitosan nanofibers. Because of decrease in collagenase activity in 80% (v/v) ethanol solution (33 % decrease compared to non-sterilized sample), we investigated the possibility of irradiation with UV-C after prior desiccation. Desiccated collagenase chitosan nanofibers did not showed significant decrease in collagenase activity. UV-C radiation of biofunctionalized nanofibers from both sides from length 1 m during 30 min was followed by collagenase activity measurement, which showed decrease in enzyme activity to 87 %. These results indicate that highly active collagenase chitosan nanofibers can be successfully sterilized by UV-C without significant decline in activity.

CONCLUSION

In this study, collagenase from *Clostridium histolyticum* was immobilized via carbodiimide chemistry to chitosan nanofibers. Immobilized microbial collagenase exhibited high affinity to Pz-peptide demonstrating that steric active site accessibility is maintained. The combination of collagenase and nanofibers served as excellent immobilization matrix properties for a long period stability by means of robust covalent bonds between collagenase and chitosan nanofibrous surfaces. Further, desiccation of hydrophilic biocompatible collagenase nanofiber followed by UV-C sterilization allows unchanged hydrolysis of peptidic substrate Pz-peptide. The approach of collagenase chitosan nanofibers can be employed for other enzyme applications such as debridement in cutaneous ulcers and burn wounds with advantages of sterile wound dressing or degrading various types of collagen and gelatine in biological methodology.

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