TESTING OF COLLAGENASE COVALENTLY LINKED TO CHITOSAN NANOFIBERS FOR BIOMEDICAL APPLICATIONS

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Abstract

Nanofibers biofunctionalized with enzymes are 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 with a microbial collagenase. The morphology and microstructure of the chitosan nanofibers prepared with Nanospider technology were examined using a scanning electron microscope (SEM). Amino groups of a chitosan nanofiber were connected with the carboxylic acid groups of the collagenase using EDAC and sulfo-NHS methodology. Collagenase enzymatic activity was evaluated with a 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. The characterisation of collagenase chitosan nanofibers in terms of measuring apparent Michaelis-Menten constants (K_M(app) and v_{max}) revealed a higher enzyme to substrate affinity than with the soluble form of the collagenase. The resulting novel collagenase chitosan nanofibers exhibited 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, such 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 the isolation of a broad variety of cell types, especially 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, a frequently used technique for the removal of necrotic tissue from wounds. Microbial collagenase is already used as an active ingredient ointment (Iruxol® Mono Ointment), where it is a safe and effective choice for the 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 a 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 needleless electrospinning as an enzyme-releasing scaffold have potential to enhance integrative repair [7]. An example of a suitable delivery system is collagenase stored inside electrospun poly(ethylene oxide) nanofibers releasing active molecules upon hydration [5]. Covalent linkages are used to effectively prevent both the denaturation and leaching of enzyme molecules [8]. In particular, immobilizing the proteases by covalent bond additionally decreases autocatalysis and controls proteolysis [9].

In this article, we describe an optimized method of covalent collagenase immobilization to electrospun chitosan nanofibers. Collagenase activity, being a fundamental proof of the enzyme occurring in its active form, was investigated using the hydrolysis of a peptidic substrate and optimized for use with the nanofiber membrane. Other characteristics such as storage and operational stability, Michaelis-Menten constant measurement are described. Desiccation as a potential storage method for collagenase chitosan nanofibers was investigated, as well as sterilization conditions.

2. EXPERIMENTAL

2.1. Chemicals and manufacture 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-peptide (4-phenylazobenzyloxycarbonyl-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 technology Nanospider™ in an NS LAB 500 S electrospinning laboratory device (Elmarco Ltd., Liberec, Czech Republic) [10]. Briefly, 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 an electrospinning methodology and were crosslinked by heating to 130°C for 1 hour. Nanofiber thickness was analysed by scanning electron microscopy (SEM) using a TESCAN VEGA3 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 torn off and all squares were weighed.

2.2. Collagenase immobilization onto chitosan nanofibers

The enzyme collagenase was immobilized onto the 1.5 × 1.5 cm chitosan squares according to [11] with slight modifications. Nanofibrous squares were rehydrated with 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. The 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 with mild rotation. Subsequent washing was carried out 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 twice 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.

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 $CaCl_2$ according to [12]. 1 U according to Wünsch catalyzes the hydrolysis of 1 µmole of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine per minute at 25 °C, pH 7.1. The resulting 4-phenylazobenzyloxycarbonyl-Pro-leucin, after its extraction with ethylacetate, changed to a yellow product which was measured spectrophotometrically. Specifically, the 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 $CaCl_2$ at 37°C (final reaction volume 2 ml). The enzymatic reaction occurred under mild stirring and was stopped after 25 min by the 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 a quartz cuvette using a Biochrom LIBRA S22 UV/VIS spectrophotometer (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 above in section 2.3. was used to acquire data for K_M(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 a 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 at 37°C. 0.24 ml aliquots were then stopped with 1 ml of 3% citric acid, and extracted with 2 ml of ethyl acetate. The change in absorbance of the organic phase at 320 nm was monitored at 3 min intervals for an overall period of 24 min in the Biochrom Libra S22 UV/VIS spectrophotometer (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 $CaCl_2$ with sodium azide (0.1% w/v) at 4°C. The 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 $CaCl_2$ (1×1 ml).

2.6. Sterilization of collagenase chitosan nanofibers

Desiccated collagenase chitosan nanofibers were irradiated with microbicidal UV-C radiation (γ = 253.7 nm) or decontaminated with 80% ethanol. The first method consists of irradiation from both sides from a distance of 1 m for 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 $CaCl_2$. The 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 3 times in 0.03 M TRIS-HCl buffer (pH 7.0) with 0.2 M NaCl and 5 mM $CaCl_2$.

3. RESULTS AND DISSCUSSION

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

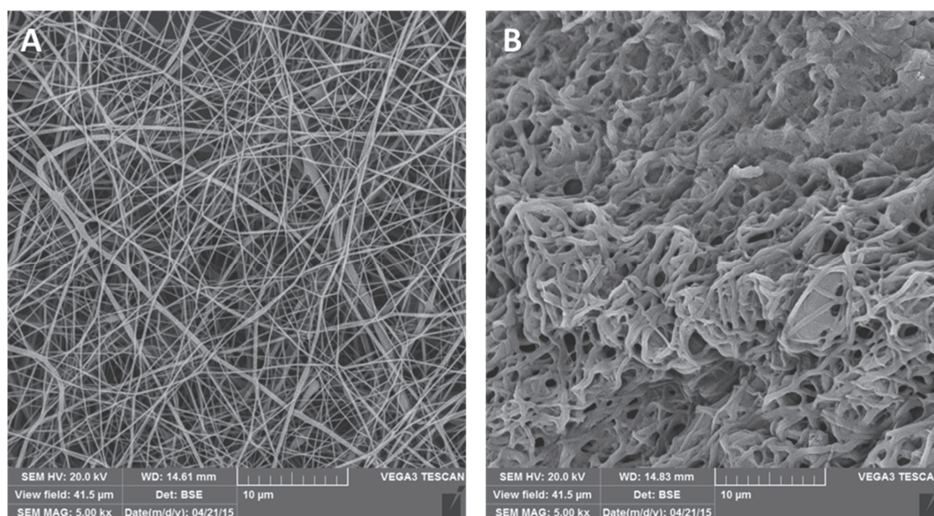

Fig. 1 Scanning electron microscope image of chitosan nanofibers before (A) and after (B) hydratation, magnification 5, 000 x. TESCAN VEGA3

significantly changed, and the nanofibrous structure was apparently preserved.

The immobilization of microbial collagenase was performed by the EDAC and sulfo-NHS method. The principle of the covalent immobilization of collagenase is based upon the activation of free carboxylic acid groups on the chitosan nanofibers 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 produce 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. Various parameters such as the amount of EDAC, s-NHS and collagenase, length of the incubation period and temperature used in the immobilization process were optimized. Using the most suitable conditions, the highest possible level of enzyme activity is shown in the plot of collagenase chitosan nanofiber activity against temperature and time of incubation (**Fig. 2**). The highest level of collagenase chitosan nanofiber activity (3.51 ug/mg nanofibers) was achieved using 4°C and 7 h of incubation. These conditions, and clearly the decreased temperature, ensured suitable conditions for low-enzyme autolysis and the highest enzyme activity yield. Furthermore, the resulting immobilized collagenase activity is fully comparable to the ointment Iruxol Mono (0.48-3 mg collagenase / g of ointment), which is available on the market [13].

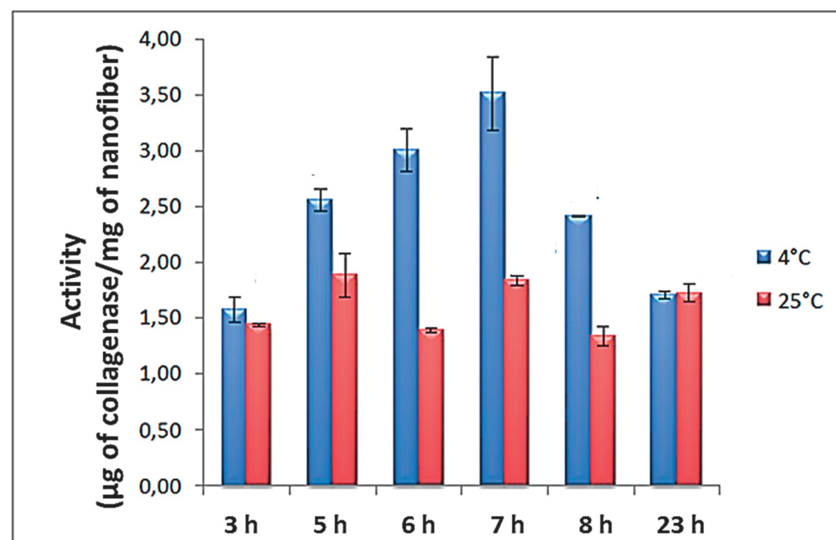

Fig. 2 Using different incubation times and temperatures for the immobilization of collagenase to chitosan nanofibers with the optimized amounts of chemicals

3.2. Characterization of collagenase chitosan nanofibers

In this study, we biochemically characterized the affinity of clostridial collagenase NB 4G from *Clostridium histolyticum* to the synthetic peptidic substrate Pz-peptide. We performed Michaelis-Menten kinetic measurements (**Table 1**). The resulting apparent Michaelis-Menten constant (K_M(app)) values are consistent with the literature [1], differences were caused by using different substrates. The kinetic parameters K_M(app) and v_{max} are especially important parameters 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(app) for collagenase immobilized onto chitosan nanofibers compared to the K_M(app) for soluble collagenase indicates a higher affinity for the active binding site of collagenase. These lower values correspond to previous observations of enzyme conjugations to materials such as magnetic particles [14] or polyethylene glycol [15]. A possible interpretation of the lower values compared to soluble collagenase is that the large specific surface area for hydrophilic nanofibers changes the degree of substrate pre-concentration in proximity to the nanofiber surface.

Table 1 Michaelis constant of soluble and immobilized collagenase

	Substrate	(K_M(app) (mM)	v_{max} (mol/l.s)	References
Collagenase (class I-II) chitosan nanofibers	4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg	0.356	0.09	This work
Soluble collagenase (class I-II)	4-phenylazobenzyloxycarbonyl-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, the operational and storage stabilities of nanofibers biofunctionalized with collagenase were observed. The operational stability measurement results (**Fig. 3**) showed no substantial decrease after measurement 8. Storage stability is one of the main advantages of enzyme immobilization. To demonstrate the long-term usability of immobilized collagenase, the prepared collagenase chitosan nanofibers were stored at 4 °C in storage buffer before the enzyme activity measurement. The results showed no significant decrease in specific enzyme activity (still at 100%) after 6 weeks.

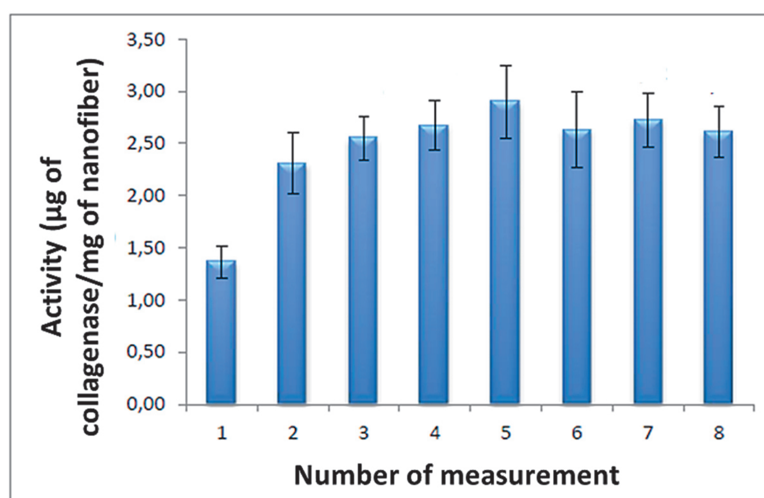

Fig. 3 Operational stability of collagenase chitosan nanofibers

3.3. Sterilization of collagenase chitosan nanofibers

We further investigated the sterilization conditions of collagenase chitosan nanofibers. Because of the 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 exhibit a significant decrease in collagenase activity. The UV-C radiation of biofunctionalized nanofibers from both sides from a distance of 1 m for 30 min was followed by collagenase activity measurement, which showed a decrease in enzyme activity to 87 %. These results indicate that highly active collagenase chitosan nanofibers can be successfully sterilized by UV-C without a significant decline in activity.

4. CONCLUSION

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

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