CHITOSAN NANOFIBERS AND NANOPARTICLES FOR IMMOBILIZATION OF MICROBIAL COLLAGENASE

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Abstract: *The purpose of the paper is to describe the production and characterization of electrospun chitosan nanofibers and chitosan/TPP nanoparticles as carriers for microbial collagenase. These proteolytic highly active and stable carriers have a potential of advanced bioactive wound dressings. The morphology and the microstructure of nanofibers and nanoparticles were examined using a scanning electron microscopy and dynamic light scattering. Low molecular weight chitosan/TPP nanoparticles prepared at the ratio 2:1 showed a size range of 130 - 142 nm. Specific collagenase activity 0.545 × 10^{-3} U/mg for chitosan nanofibers and 0.684 × 10^{-3} U/mg of nanoparticles was quantified using the hydrolysis of a peptidic substrate (Pz-peptide) and is expressed per mass of chitosan biomaterial. Storage stability and reusability of collagenase chitosan nanofibers and collagenase chitosan nanoparticles did not fall below 73% of their original activity.*

Key Words: *chitosan, nanofibers, nanoparticles, collagenase, stability*

1 INTRODUCTION

Chitosan (CS) is a derivative of chitin and is a natural polyaminosacharide offering a set of unique characteristics that include biocompatibility, biodegradability, nontoxicity, antibacterial properties, and hydrophilicity [1]. In the past decades, CS has been extensively used as a carrier candidate in biomedicine because of its excellent stability, mucoadhesive property, and being a suitable carrier of nucleic acids, enzymes and antigens [2-6].

The nanofibrous form of CS brings additional attractive features like large surface area and porosity leading to good permeability for oxygen and water [7, 8]. These properties support cell respiration, skin regeneration, moisture retention, removal of exudates, and hemostasis [9]. The formation of CS nanoparticles is a process based on the complexation of oppositely charged molecules by electrostatic interactions [10]. Nanoparticles can easily be constructed using an ionic gelation process which involves sodium tripolyphosphate as a negatively charged molecule and CS as polycation [11, 12]. Low molecular weight chitosan is popular primarily due to its better solubility in the neutral aqueous solvents with low viscosity and rapid degradation than is chitosan having a higher molecular weight [2, 13].

Nanofibrous scaffolds fabricated via electrospinning represent a potential delivery vehicle. 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 [14]. An example of a suitable delivery system is collagenase stored inside electrospun poly(ethylene oxide) nanofibers releasing active molecules upon hydration [15]. 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 [16]. 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 [17-19].

This work describes the production and characterization of electrospun chitosan nanofibers and chitosan nanoparticles as carriers for microbial collagenase. Covalent linkage used in this work is used to effectively prevent both the denaturation and leaching of enzyme molecules [20]. In particular, immobilizing the proteases by covalent bond additionally decreases autocatalysis and controls proteolysis [21, 22]. Carbodiimide chemistry for grafting the collagenase from *Clostridium histolyticum* was used. Both chitosan nanofibers and nanoparticles were compared in terms of immobilized collagenase activity, stability and reusability. Collagenase activity, being a fundamental proof of the enzyme

occurring in its active form, was quantified using the hydrolysis of a peptidic substrate.

2 EXPERIMENTAL

Chemicals

KiOnutrime-CS (Kitozyme, Belgium), polyethylene oxide (Scientific Polymer Products, NY, USA), chitosan I (MW 47 kDa, deacetylation 80%) (Contipro, Dolni Dobrouc), 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, sodium tripolyphosphate (TPP) and other pure chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Manufacturing the nanofibers

CS nanofibers, described in detail in [23], were prepared using the modified needleless technology NanospiderTM in an NS LAB 500 S electrospinning laboratory device (Nanovia Ltd. Litvínov, Czech Republic) [24]. Briefly, CS nanofibers were prepared from the polymer solution of CS (5 wt% to 20 wt%) and polyethylene oxide (1 wt% to 10 wt%), in acetic acid by an electrospinning methodology and were crosslinked by heating to 130°C for 1 hour.

Chitosan/tripolyphosphate (CS/TPP) nanoparticles preparation under stirring according to [Biswas]. 15 mL of 2.3 mM TPP was mixed with 30 mL of 0.2% CS in 25 mM acetate buffer pH 5.2 (CS:TPP ratio 2:1) for 30 minutes at room temperature. Dialysis followed using the 3.5 kDa molecular weight cut-off dialysis tubing (Serva Electrophoresis) to 0.1 M acetate buffer pH 5.5. For further analysis, the nanoparticles were stored at 4°C and 0.1 M acetate buffer pH 5.5 or lyophilized from distilled water using the Scan Vac CoolSafe (Labogene, Denmark).

Dynamic light scattering

The average particle size and size distribution of the prepared nanoparticles in different media were determined by dynamic light scattering (DLS) using a Zetasizer and software 7.03 version (Malvern Instruments Ltd., UK). The measurements were performed at 25°C, with a scattering angle of 173°, using disposable cuvettes. Amount of nanoparticles per sample was 0.25 mg in 1.5 mL. Each measurement was performed in 12 repeats after 30 s. Data were statistically processed.

Scanning electron microscopy

Scanning electron microscopy of prepared particles was performed using VEGA3 SBU apparatus and backscattered electron detector (Tescan, Czech Republic). Samples were coated with a conductive layer of gold (thickness of 0.2 nm) in Balzers sputter coater to prevent charging of the specimen.

Collagenase immobilization

The enzyme collagenase was immobilized onto 1.5×1.5 cm CS squares or 2 mg of CS/TPP nanoparticles according to [23]. Briefly, 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. All measurements were repeated a minimum of two times the calculated means and SD values of which are shown in the graphs.

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 [25]. 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).

3 RESULTS AND DISCUSSION

In this study, a strategy for covalent biofunctionalization of CS nanofibers and CS/TPP nanoparticles with collagenase from *Clostridium histolyticum* is presented. The work was carried out

with the aim to prepare proteolytic highly active and stable nanofibers with the potential of advanced bioactive wound dressings.

For the preparation of the nanofibers we used modified needleless Nanospider™ technology [26]. This technology enables flexibly the formation of fibers tens of nanometers to tens of micrometers in diameter and the preparation of nanofibers with masses per unit area ranging from 1 to 100 g/m^2. Nanofiber structure was analysed by scanning electron microscopy (Figure 1) using a TESCAN VEGA3 microscope.

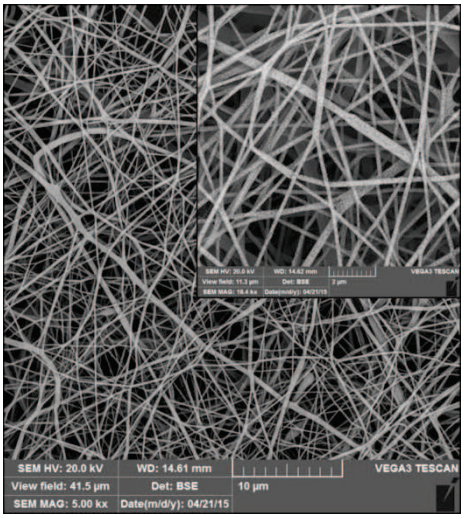

Figure 1 Scanning electron microscopy of CS nanofibers. Magnification 5,000× and 18,000× for inset. VEGA3 TESCAN instrument

CS/TPP nanoparticles were prepared according to the procedure first reported by Calvo et al. [11] with suitable modifications based on the ionotropic gelation of CS with TPP anions [2]. To achieve the nanoparticle sizes around a hundred nm, different ratios of CS to TPP were carried out and the composition of this optimisation was chosen as CS/TPP ratio 2:1. The hydrodynamic diameter of the prepared CS/TPP nanoparticles dialyzed against the same buffer was determined by the means of dynamic light scattering (DLS). Resulting hydrodynamic sizes (Table 1) were close for the CS/TPP nanoparticles freshly prepared (sample **1**) and those dialyzed to 0.1 M acetate buffer pH 5.5 (sample **2**).

Furthermore, the effect of the storage to the nanoparticles was monitored using the DLS and size distribution. Results on Figure 2 and Table 1 show that nanoparticles stored at the conditions of 4°C and 0.1 M acetate buffer pH 5.5 did not change in hydrodynamic size (sample **3**). Size distribution also remained unchanged.

Table 1 Results of DLS measurement of CS/TPP nanoparticles (**1 - 3**). Concentration of sample particles 16.67%, vortex before measurement, 25°C, 0.1 M acetate buffer pH 5.5

Particle sample	Mean D_H (nm)	SD
(**1**) CS/TPP nanoparticles, no dialysis	132.4	1.57
(**2**) CS/TPP nanoparticles, dialysis	139.3	1.78
(**3**) CS/TPP nanoparticles, stored for 7 weeks, dialysis	142.6	1.31

The morphology of the CS/TPP nanoparticle lyophilized from distilled water studied from scanning electron microscopy images (Figure 3) revealed that the nanoparticles have particle character. During application and gilding lyophilized sample on a slide pad, distinct formation of aggregates with a porous structure takes place.

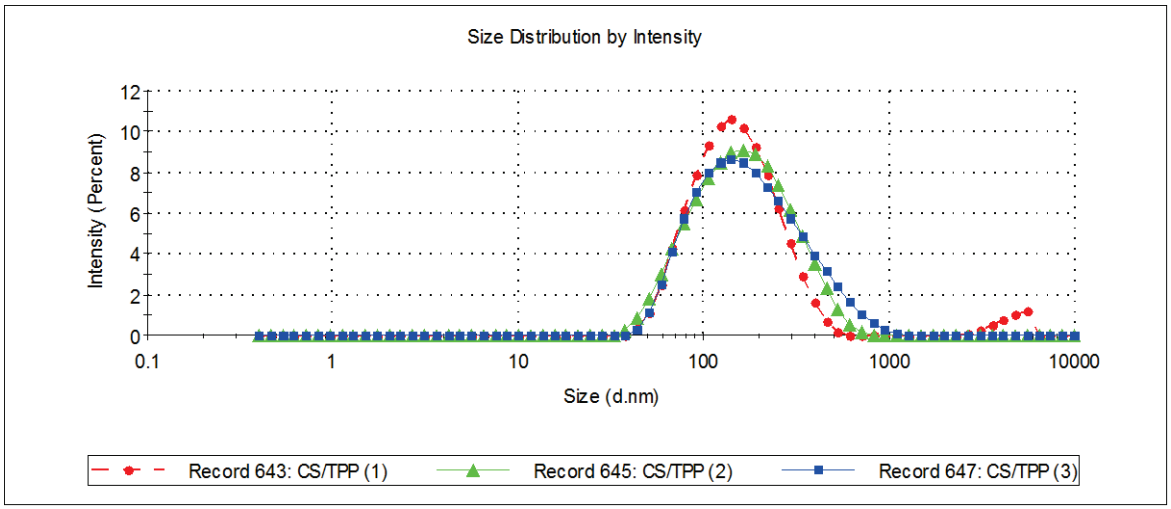

Figure 2 Size distributions by intensity of samples (**1-3**) of CS/TPP nanoparticles (circle – no dialysis (**1**), triangles – dialysis (**2**), storage 7 weeks and dialysis (**3**))

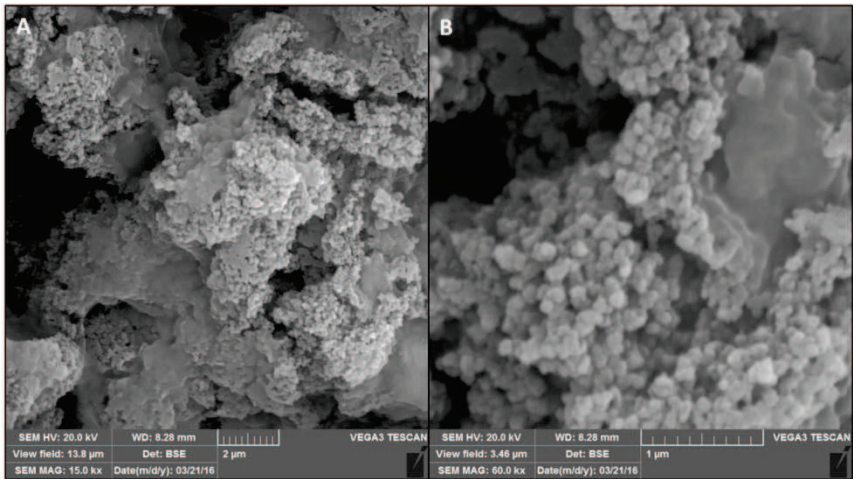

Figure 3 Scanning electron micrograph of CS/TPP nanoparticles. Magnification **A)** 15,000×, **B)** 60,000×

Immobilization method applied in this study is based on the formation of amide and ester linkages mediated by the zero-length crosslinker carbodiimide (EDC). The addition of sulfo-NHS to the EDC-mediated reaction increases the solubility and stability of the active intermediate O-acylisourea subsequently reacting with the amino and hydroxyl group of chitosan. EDC/sulfo-NHS-coupled reactions are generally considered highly efficient [27]. CS nanofibers of basis weight 20 (g/m^2) 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. Dialyzed CS/TPP nanoparticle suspensions (2 mg of solid) were washed and supernatants were removed before carbodiimide immobilization method of the collagenase occurred. The collagenase CS nanoparticles were sonicated for 10 minutes before immobilization and activity assays. Specific enzyme activity in units is expressed per mass of CS biomaterial (Table 2). The resulting values for immobilized collagenase activities on nanofibers are lower by 20.3% compared to the activity of the enzyme on nanoparticles. Possible explanation for the difference is a different form, size, or specific surface.

Table 2 Immobilized collagenase activity

Particle sample	Specific enzyme activity (U/mg of biomaterial)
Collagenase CS nanofibers	0.545×10^{-3}
Collagenase CS/TPP nanoparticles	0.684×10^{-3}

Storage stability and reusability are the main advantages of immobilized enzymes. This also testifies about the quality of the binding of the enzyme to the carrier. To demonstrate the long-term usability of immobilized collagenase, the prepared collagenase CS nanofibers and collagenase CS nanoparticles were stored at 4°C in 30 mM Tris-HCl buffer pH 7.0 with 0.2 M NaCl a 5 mM $CaCl_2$ and monitored for 4 weeks. The collagenase CS nanoparticles were sonicated for 10 minutes before activity assay. The results of specific enzyme activity for collagenase CS nanofibers showed no decrease (still at 100%) after 4 weeks, collagenase CS nanoparticles activities decrease to 73% of original values (Figure 4). An increase in collagenase CS nanofibers activity recorded after 1 week of storage is a commonly observed phenomenon caused by the restoration of functional conformation leading to enzyme reactivation [28]. These experiments repeatedly confirmed high and sufficient proteolytic activity of stored collagenase CS nanofibers and CS/TPP nanoparticles.

The reusability of collagenase CS nanofibers and CS/TPP nanoparticles were measured 8 times in a row during one day. Excellent results of collagenase CS nanofibers were observed (Figure 5), activity per mg of nanofibers did not decrease below 100%. Collagenase CS/TPP nanoparticles activity after the last run did not fall below 78% of its original value. These results show the ability of immobilized collagenase to repeatedly cleave the target substrate without any substantial activity loss.

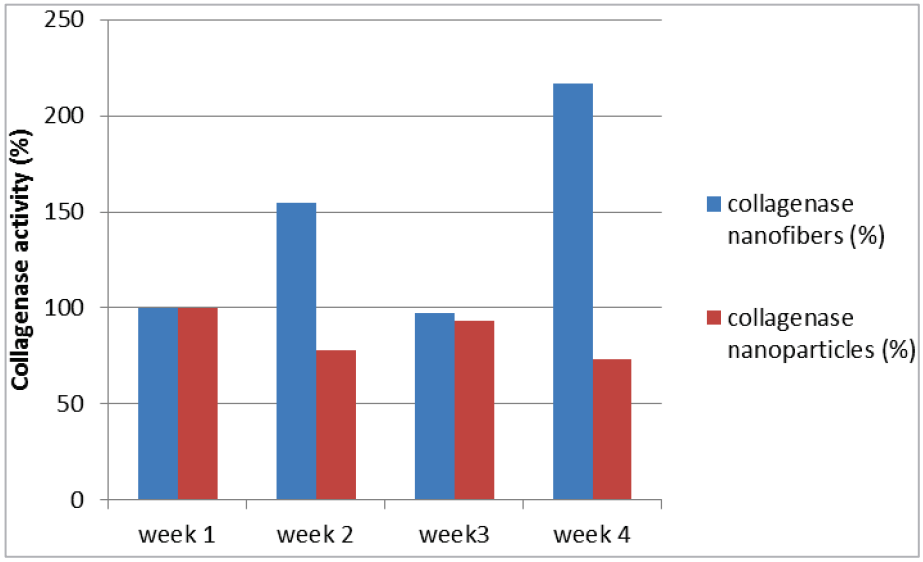

Figure 4 Storage stability of immobilized collagenase on CS nanofibers and on nanoparticles

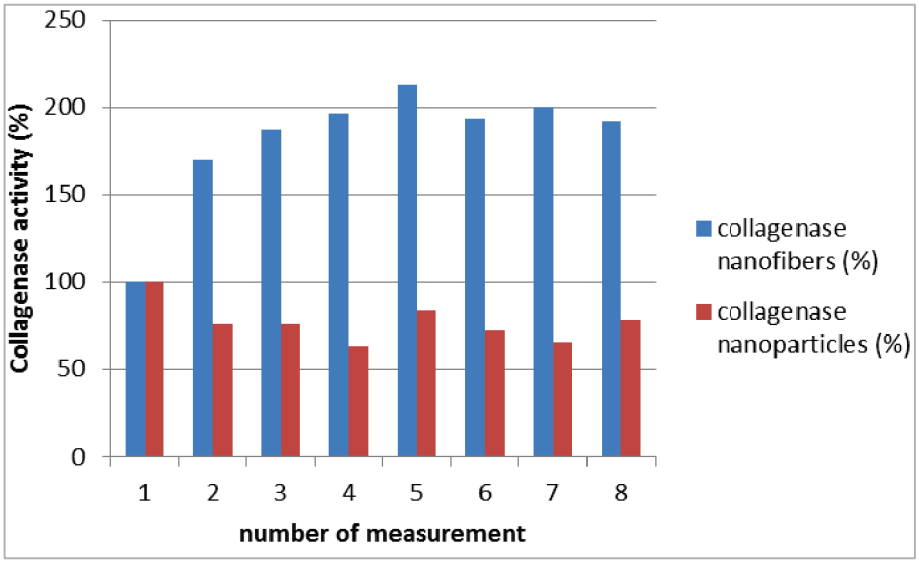

Figure 5 Reusability of immobilized collagenase on CS nanofibers and on nanoparticles

4 CONCLUSIONS

In this work, biocompatible chitosan electrospun nanofibers and chitosan/tripolyphosphate nanoparticles were biofunctionalized with the enzyme collagenase from *Clostridium histolyticum*. In comparison, higher collagenase activity per mg was achieved when using the chitosan/TPP nanoparticles.

This resulted in an activity of up to 0.545×10^{-3} U/mg of nanoparticles and 0.684×10^{-3} U/mg of nanofibers, respectively. This study confirmed comparable results of chitosan biomaterials in its different forms. The excellent stability and reusability of collagenase immobilized on chitosan carriers suggests their potential in enzyme debridement for skin regeneration.

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