IMMOBILIZATION OF ENZYMES ON THE SILICA NANOFIBERS FOR BURNS TREATMENT

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Abstract

For burns and necrotic wounds, removal of the necrotic tissue is the first step to successful treatment. Effective wound cleansing and debridement are essential for granulation and re-epithalisation. Now it is possible to replace a current very painful surgical removal with the enzymatic or autolytic debridement. Especially enzymatic debridement is a highly selective method that utilizes naturally occurring proteolytic enzymes. The reasons why the surgical removal has not yet been replaced by painless enzymatic debridement are low enzyme stability and many factors affecting enzymes activity (type of substrate, pH, temperature, etc.).

The possibility to solve this problem is the enzyme immobilization on the suitable material - e.g. nanofibers with a lot of functional groups for covalent binding of the enzymes. As a supporting biocompatible and biodegradable material for immobilization of several enzymes types, the electrospun silica nanofibers were chosen. Proteolytic activity of immobilized enzymes was tested under different conditions.

Keywords: Silica nanofibers, proteolytic enzymes, burns, immobilization

1. INTRODUCTION

Deep partial thickness and full thickness of burns are characterized by the presence of necrotic tissue - the eschar. The eschar makes accurate diagnosis of burn depth difficult and contributes to local and systemic complications. Therefore, early burn eschar removal (debridement) is essential for the proper treatment of burns [1]. The most commonly used surgical debridement is very painful and damages the surrounding healthy skin tissue. Besides surgical removal, painless types of debridement exist - autolytic or enzymatic debridement, which don't cause further wound damage. Especially proteolytic enzymes are chosen for enzymatic debridement, because they catalyze proteolytic reactions (hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain). Enzymes digest a necrotic tissue. Proteolytic enzymes are already used in the treatment of burns; however, they are usually mixed in gels or ointments (e.g. mixing of NexoBridTM with bromelain in an inert carrier gel [2], Iruxol Mono with collagenase from *Clostridium histolyticum* in white petrolatum [3] or Accuzyme oitment with papain-urea [4].

Low stability of the enzymes, their temperature dependence and pH dependence are restrictions, why they aren't commonly used for painless treatment of burns. Silica nanofibers appear to be a very good material for the immobilization of enzymes on silica surface. They are biocompatible, biodegradable and they have a number of active functional groups for covalent binding of enzymes. Electrospun nanofibers are structurally similar to the extracellular matrix and they are able to support the epithalisation of new skin tissue after necrosis removal.

For immobilization of proteolytic enzyme, it is necessary to functionalized silica nanofibers surface. As a silanization reagent, 3-Aminopropyl triethoxysilane was chosen. For carboxylation of amino groups of 3-Aminopropyl triethoxysilane, succinic anhydride was used. The final step was reaction of mentioned chemicals with N-Hydroxysuccinimide ester.

After functionalization of silica nanofibers, following proteolytic enzymes were immobilized on silica nanofibers: protease from *Aspergillus oryzae,* trypsin from hog pankreas and bromelain from pineapple stem. Their proteolytic activity was measured by using casein as a substrate. This research examines the temperature influence on the activity of immobilized proteolytic enzymes on the silica nanofibers.

2. EXPERIMENT

2.1. Material

Nanofibers were prepared from tetraethyl orthosilicate (TEOS, Sigma-Aldrich, 98 wt.%), propan-2-ol (Penta CZ, p.a. 99.9 wt.%), distilled water, hydrochloric acid (Sigma-Aldrich-Fluka, 2 mol/L). For functionalization of the nanofibers surface, ethanol absolute (Penta) and the following chemicals and enzymes purchased in Sigma-Aldrich were used: 3-Aminopropyl triethoxysilane (APTES, 98 wt.%), succinic anhydride (SU, 99 wt.%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98 wt.%), N-Hydroxysuccinimide (NHS, 98 wt.%), 4-Morpholineethanesulfonic acid monohydrate (MES, 99 wt.%), Tris(hydroxymethyl)aminomethane (TRIS, 99.8 wt.%), protease from *Aspergillus oryzae* (4.3 U/mg), trypsin from hog pankreas (1670 U/mg) and bromelain from pineapple stem (3.2 U/mg). For enzyme activity assay, casein from bovine milk and tricloracetic acid (TCA, 99 wt.%) were used (both Sigma-Aldrich).

2.2. Preparation of Silica Nanofibers

Silica nanofibers were prepared according to [5] by sol-gel method and subseqently electrospun. TEOS (300 mL) and propan-2-ol (330 mL) were added into an Erlenmeyer flask and mixed together for 15 minutes at ambient temperature. Subsequently, a solution of propan-2-ol (200 mL), 2M HCl (8 mL) and distilled water (60 mL) was prepared and added into the mixture under continuous stirring. In the next step, the solution was gently cooled to ambient temperature and then was stirred again, refluxed and heated in an oil bath (temperature 200 °C) for 2 hours. Finally, the propan-2-ol was distilled off the prepared sol.

Silica nanofibers were electrospun from free liquid surface on the needleless NanospiderTM device (Elmarco). Electrospinning process ran under standardized conditions (electrode distance: 140 mm, string speed: 0.2 mm/s, head speed: 350 mm/s in 500 mm, tow: 90/100m^3/h, voltage: 57 kV, air humidity 33 %, laboratory temperature 23 °C). Silica nanofibrous sheet of specific weight (32 $g.m^{-2}$) and mean fiber diameter (approximately 200 - 700 nm) was obtained. Subsequent thermal stabilization of the silica nanofibers was performed at 180 °C for 2 hours [6].

2.3. Functionalization of the Pure Silica Nanofibers

For silanization reaction of pure silica nanofibers, 2% APTES in propan-2-ol and distilled water (for 1 hour at room temperature) was applied. The silica nanofibers were rinsed with distilled water twice. Succinic anhydride was chosen as a reagent for carboxylation of amino groups from APTES. SU was dissolved in absolute ethanol with concentration of 2 mg/ml. The samples were then immersed into the prepared solution at ambient temperature for 2 hour. NHS ester was created using EDC and NHS. This ester reacted with the carboxyl groups of SU at one end and with amino group of enzymes at the other end (**Figure 1**). The zero-length crosslinker EDC (1 mg/ml) and NHS (1.25 mg/ml) reagent (each dissolved in 0.1 M MES buffer with pH=6) were quickly added to the nanofibers (20 minuts, room temperature, shaking). After fuctionalization, nanofibers were rinsed twice with MES buffer. The nanofibers were divided into three parts for immobilization of trypsin, bromelain and protease enzyme.

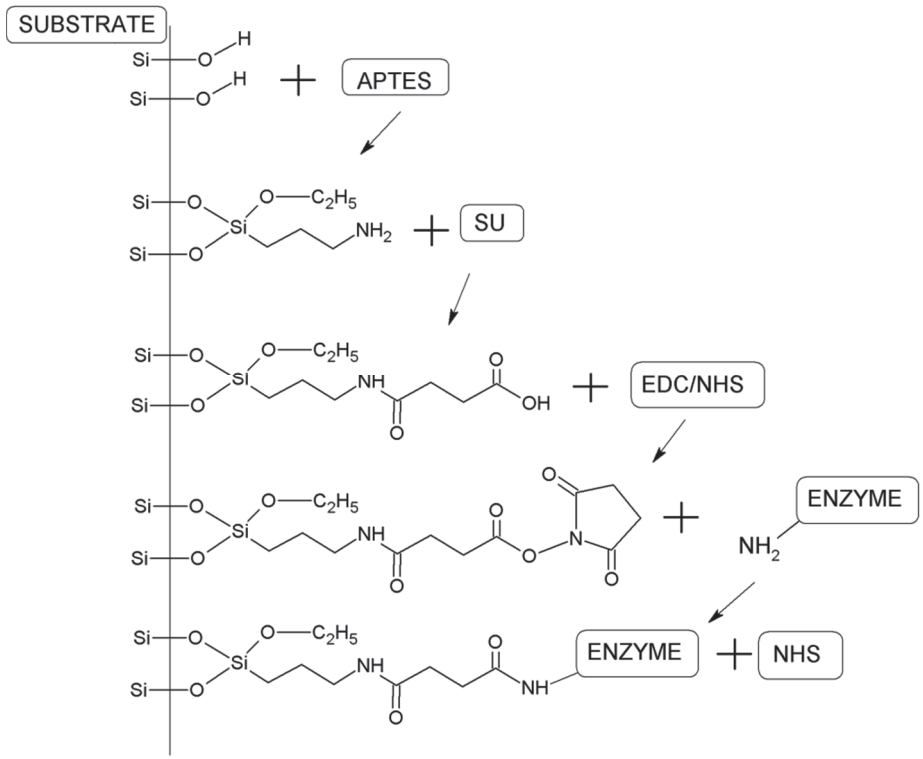

Figure 1 The scheme of the enzyme immobilization on the silica nanofibers

2.4. Immobilization of the Enzymes on Functionalized Silica Nanofibers

Protease from *Aspergillus oryzae*, trypsin from hog pankreas and bromelain from pineapple stem were immobilized on functionalized silica nanofibers as a model of proteolytic enzymes. Protease was chosen as type of the bacterial proteolytic enzyme, trypsin as animal enzyme and bromelain was chosen as a typical vegetal proteolytic enzyme. Each of these enzymes was separately dissolved in 0.1 M phosphate buffer with pH=7.3 in a concentration of 10 mg enzymes per ml. Immobilization of enzymes on silica nanofibers was carried out for 2 hours under gentle shaking at room temperature. Then, the nanofibers were rinsed once in the phosphate buffer. The final step was reaction of unreacted NHS esters with 30 mM TRIS in distilled water. Samples (cca 1x1 cm) were slowly dried at 36 °C for 3 hours and weighed.

2.5. Visualization of Nanofibers by Scanning Electron Microscope - SEM

Images were taken with the scanning electron microscope Carl Zeiss ULTRA Plus. The samples (magnified 25000x) were gold-dusted in advance and observed (through the In-Lens detector) in the form of secondary electrons SE1.

2.6. Enzyme Activity Assay

Proteolytic assays of immobilized enzymes were performed using casein as substrate. Caseinolytic activity was measured at three different temperatures: 4, 23 and 37 °C. The nanofiber sample with immobilized enzyme was inserted to the casein in MES buffer (0.65 %, pH = 5.5). The enzyme-casein reaction was stopped after 20 min by addition of TCA (110 mM). This solution was incubated for 30 min. Then each of samples was filtered by using a 0.45 µm polyethersulfone syringe filter. To the defined volume of filtrate, 500 mM of sodium carbonate solution and 0.5 M of Folin's reagent were added [7]. The reaction was carried out for 30 min and then absorbance was measured at 750 nm.

3. RESULTS AND DISCUSSION

Silica nanofibers were visualized by SEM and proteolytic activity was measured by Lowry method with minor modifications at 4, 23 and 37 °C.

Selected SEM images of the silica nanofibers with and without immobilized trypsin enzyme are shown in **Figures 2A** and **2B**. In the case of the trypsin enzyme immobilization, the surface of the silica nanofibers changed and was not smooth (see **Figure 2B** at a magnification of 25000x) - there were clearly visible structures covering the whole surface of the silica nanofibers continuously. In the case of non-modified silica nanofibers (**Figure 2A**), we can clearly see a smooth surface without any artefacts. The nanofibers have different thickness in both cases (**Figures 2A, 2B**).

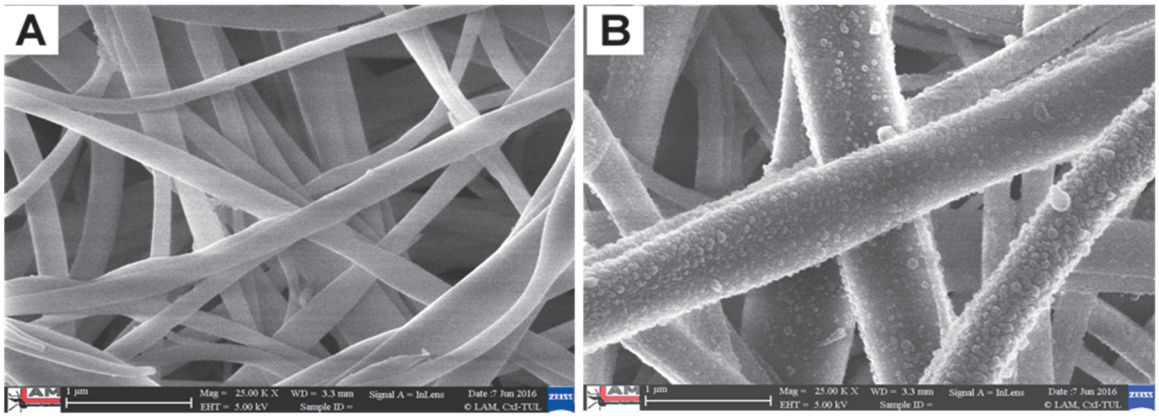

Figure 2 SEM images of non-modified silica nanofibers (A) and silica nanofibers with immobilized trypsin enzyme (B)

Proteolytic activity of immobilized trypsin, bromelain and protease on the silica nanofibers was measured at pH = 5.5 by the method described above. The pH value of 5.5 was chosen because the pH of skin ranges from about 4.8 to 6.0. Because the amount of immobilized enzyme was unknown, the proteolytic activity was converted to micrograms of enzyme per miligrams of nanofibers. The results are shown in **Figure 3**. Each of samples was prepared and measured in triplet.

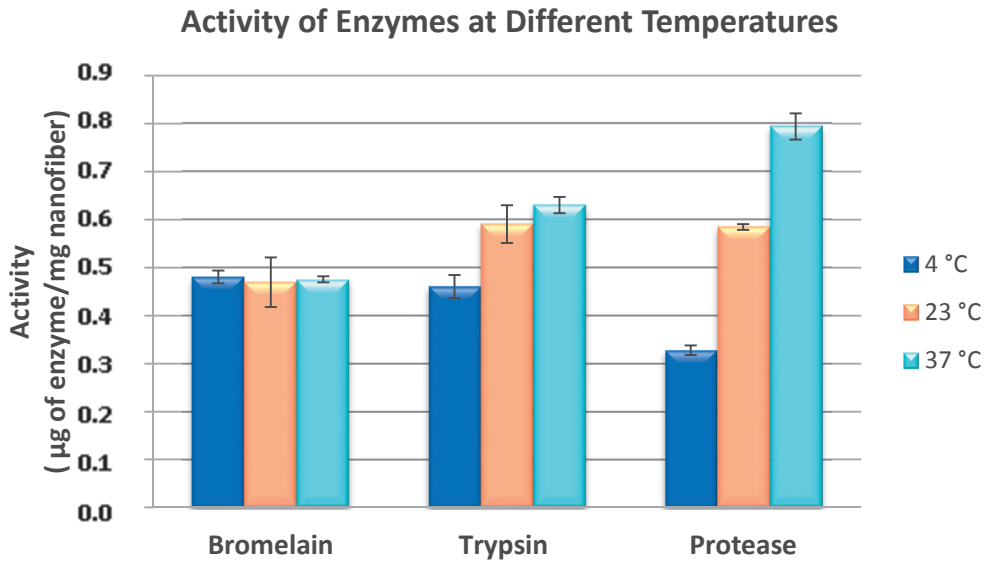

Figure 3 Activity of the immobilized bromelain, trypsin and protease at the different temperatures

Effect of temperature on enzyme activity was observed in the case of trypsin and protease. Higher temperature influence on the proteolytic activity was demonstrated for nanofibers with the protease where the difference between the activity at 4 °C and 37 °C was more than doubled. Conversely, bromelain seemed to be temperature independent in this temperature range. For the temperature of human body (37 °C), the highest activity was measured for the nanofibers with immobilized protease, therefore this sample has potential to be the most effective for necrotic tissue removal.

4. CONCLUSION

In this study, bromelain, trypsin and protease were immobilized on the silica nanofibers through APTES, succinic anhydride and NHS ester. Proteolytic activity of the immobilized enzymes was measured at pH = 5.5 by casein as a substrate. The effect of temperature on the immobilized proteolytic enzymes activity was tested at three different temperatures (4, 23 and 37 °C).

All enzymes retained their activity after immobilization on the silica nanofibers. The highest temperature dependence was demonstrated in the case of immobilized protease from *Aspergillus oryzae* on the silica nanofibers. These nanofibers also showed the highest proteolytic activity at 37 °C.

Because pH value of damaged skin is slighty higher than pH value of intact skin, it is necesarry to test immobilized enzymes activity for other specific pH values. It is also planned to replace the succinic anhydride with another more biocompatible heterobifunctional reagent.

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