INORGANIC SILICA NANOFIBERS IN A ROLE OF BIOACTIVE COMPOUND CARRIER

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Abstract

Nanofibers are valued for their ultra-high specific surface areas and have been found potentially useful in many biomedical applications. Modification or functionalization of nanofibers is necessary in order to engineer specific features that will help to maximize their end-use performance. A spectrum of bioactive molecules, including antibacterial agents, anti-cancer drugs, enzymes, proteins, can be incorporated into/onto nanofibers via different approaches [1].

We have focused on enzyme immobilization providing many advantages over use of its soluble form: enhanced enzyme activity, reusability, simplified processing, better storage properties and pH stability [2].

Here we describe the immobilization of trypsin (EC 3.4.21.4) on silica nanofibers. After silanisation incorporating 72 ± 12.6 μg NH_2 per mg of sheets, trypsin was bound using combinatory chemistry of EDAC and sulfo-NHS. The amount (215 ± 29.8 μg protein/cm^2) and activity (24.8 ± 2.7 I.U./cm^2) of immobilized biocatalyst as well as storage and operational stability were measured. The proteolytic activity of immobilized trypsin determined immediately after immobilization and during one week was high and stable.

Prepared bio-functional nanofiber material can find the application in wound healing process (enzyme debridement).

Keywords: inorganic nanofibers, electrospinning, trypsin, immobilization

1. INTRODUCTION

Recent advances in nano and hybrid technology have provided various nanoscale materials with high potential in many fields of research. The benefit of their use has been shown in refining electronic devices [3], efficacy scale up of filtration [4] and water remediation processes [5], accessibility of food bioengineering [6] or biomedical fine approaches [7, 8]. Huge number of these nano-applications is based on the nanomaterials capacity to bind an increased amount of bioactive molecules thanks to the higher specific surface area compared to the conventionally produced supports. Besides, engineered nano systems and native biomolecules are known to exhibit significant compatibility in terms of function, size and physicochemical properties such as pore size, aquaphilicity, surface chemistry and hydrophilic/hydrophobic balance [9-11].

Among the vast number of bioactive compounds, enzymes are of the special interest because of their crucial importance in pharmaceutical and food industries, or their high potential in filtration techniques, bio sensing and imaging trends. Problems with the loose of activity, limited selectivity and difficult recovery of the soluble biocatalyst when accomplishing its catalytic cycle are often overcome by its immobilization. Nanostructured materials with ability to control size and shape enables better interaction with the enzyme, increases immobilization efficiency, and enhances the long-term storage and recycling stability of the enzyme [12]. However, several criteria must be respected when choosing the enzyme carrier to optimize and enhance its activity when loaded on the surface [13]. Concerning the nature of the nanostructured carrier, inorganic

supports present the material of choice in this field. Their surface is suitable for common chemical modifications necessary for subsequent immobilization techniques, the possibility to control pore sizes often results in improved enzyme loading [14]. Inorganic supports as silica, titania or other oxides also present excellent thermal, mechanical and microbial resistance [15].

According to the mentioned knowledge, this paper presents the enzyme immobilization on the silica nanofibers surface modified by silanisation which introduces functional amino groups. Trypsin was chosen as a model enzyme. The surface characterization of silica nano-support was realized before and after the immobilization. The operational and the storage stability of the enzyme were measured to show the benefit of the immobilization technique.

2. MATERIALS AND METHODS

2.1 Chemicals

Trypsin (EC 3.4.21.4), 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDAC), N-a-benzoyl-D,L-arginine-p-nitroanilide (BAPNA) (EC Number 213-011-2), benzamidine, N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS), fluorescein isothiocyanate (FITC), tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA USA). The remaining chemicals were supplied by Penta (Prague, Czech Republic) and were of analytical reagent grade.

2.2 Silica nanofibers preparation

The silica nano fibrous sheets of basic weight of 240 g/m^2 were electrospun at room temperature using the NanoSpider device (NS 1WS500U, Elmarco Ltd.). The distance from the electrode to the collector was 175 mm. Voltage of 70kV was applied on the spinning solution prepared by sol-gel method from TEOS as a precursor. Electro-spun sheets were thermally treated (180°C) to ensure their prolonged stability and used for further operations.

2.3 Characterization of electrospun nanofibers

Scanning electron microscopy, SEM (Vega3 SB, TESCAN Ltd.), was used to analyse silica nanofibers prior and after the immobilization process. Samples were coated with 5nm Au/Pd using a sputter coater equipment (SC7620 Mini Sputter Coater, Quorum Technologies Ltd.). Average fiber diameters and standard deviation (SD) were determined from SEM images using VegaTC software from at least 50 randomly chosen measurements.

2.4 Silanisation of silica nanofibers using APTES

Silica nanofibers were immersed to 3% (v/v) APTES in the solution containing 4% of water in ethanol (v/v) and the pH was adjusted to 4.5 - 5.5 with acetic acid. After silanisation of nanofibers under continual shaking at laboratory temperature overnight, the samples were washed by the solution containing 4% of water in ethanol (v/v). Finally the nanofibers were dried at 110°C/30min.

2.5 Quantification of primary amine groups on silica nanofibers introduced by silanisation

Quantification of primary amine groups was realized according to the work of Ritter H. et al. (2009) [16] with some modifications. The 3.239mM stock solution of fluorescein isothiocyanate (FITC) in ethanol was prepared. Silica nanofibers were immersed to 25 fold diluted (130μM) stock solution of FITC and incubated overnight in dark, under gentle shaking. After removing the FITC solution and appropriate washing of nanofibers with ethanol, 0.2M NaOH was added and reaction mixtures were incubated in dark under vigorous shaking until nanofibers dissolution. Fluorescence of prepared solution was measured at Fex: 485/20nm, Fem: 528/20nm using Multi-Mode Microplate Reader (BioTek Instruments, USA).

2.6 Immobilization of trypsin on silica nanofibers

Immobilization was achieved through EDAC and sulfo-NHS activation as described previously [17]. Activity of the immobilized enzyme was determined using the low molecular-mass substrate BAPNA (see 2.8.).

2.7 Determination of protein (trypsin) content on silica nanofibers using bicinchoninic acid (BCA)

The amount of protein was determined using the Pierce BCA Protein Assay Kit. Microplate procedure was realized according to the instructions of manufacturer, the sample to working reagent ratio being held at 1:8. The reaction was effected under gentle shaking.

2.8 Determination of enzyme activity using low molecular-mass substrate N-a-benzoyl-D, L-arginine-p-nitroanilide (BAPNA)

Soluble or immobilized enzyme (0.1 ml) was added to 1ml of 0.1M Tris-HCl buffer (pH 7.8) with 0.025M $CaCl_2$ and 0.02 ml of 0.055M BAPNA in N,N-dimethylformamide. After incubation for 30min at 37°C reaction was stopped with 0.2 ml of 30% (v/v) acetic acid. Using Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments, USA) absorbance at 405nm was measured.

3. RESULTS AND DISCUSSION

Silica nanofibers themselves do not dispose the suitable functional groups for covalent enzyme binding. The silanisation step using APTES was required in order to introduce the primary amine functional groups on the nanofibers surface. Evaluation of silanisation process was done through the direct quantification of primary amine groups on the surface of nanofibers using FITC (see 2. 5.). The method is based on the reaction of amine groups with FITC which is subsequently detected by fluorescence measuring. The results revealed that the silanisation step have provided incorporation of 72 ± 12.6 µg NH_2 per mg of sheets.

Introduced NH_2 functional groups enabled covalent immobilization of enzyme trypsin on the surface of nanofibers through very popular crosslinker EDAC along with sulfo-NHS. These are the type of the smallest available reagent systems for bioconjugation, so-called zero-length crosslinkers. These compounds mediate the conjugation of two molecules by forming a bond containing no additional atoms. Thus, one atom of a molecule is covalently attached to an atom of a second one with no intervention of linker or spacer. The application of EDAC in particle and surface conjugation procedures along with NHS or sulfo-NHS is nearly universal and this fact makes it the most common bioconjugation reagent in use today. Both the reagent itself and the isourea formed as the by-product of the crosslinking reaction are water-soluble and may be removed easily by dialysis or gel filtration [18].

Using combinatory chemistry of EDAC and sulfo-NHS the enzyme trypsin was bound on prepared silica nanofibers. For the evaluation of immobilized active trypsin molecules the amount of protein (249.17 ± 66.5 µg/cm^2) and proteolytic activity of trypsin (9.51 ± 2.61 I.U./cm^2) were determined. The results indicate that 0.4% of enzyme molecules are fully active, neither the native enzyme conformation was not damaged nor the right orientation of enzyme molecules by immobilization was not influenced. Further immobilization techniques of trypsin need to be searched to achieve higher enzyme loading.

As Fig. 1 shows, SEM analysis confirmed no changes in the nanofibrous structure within the enzyme immobilization. High porosity of the material was preserved as well. We observed statistically non-significant (ANOVA, $p>0.05$) increase in the mean diameter of fibres prior (163 ± 61 nm) and after the immobilization (181 ± 67 nm) process.

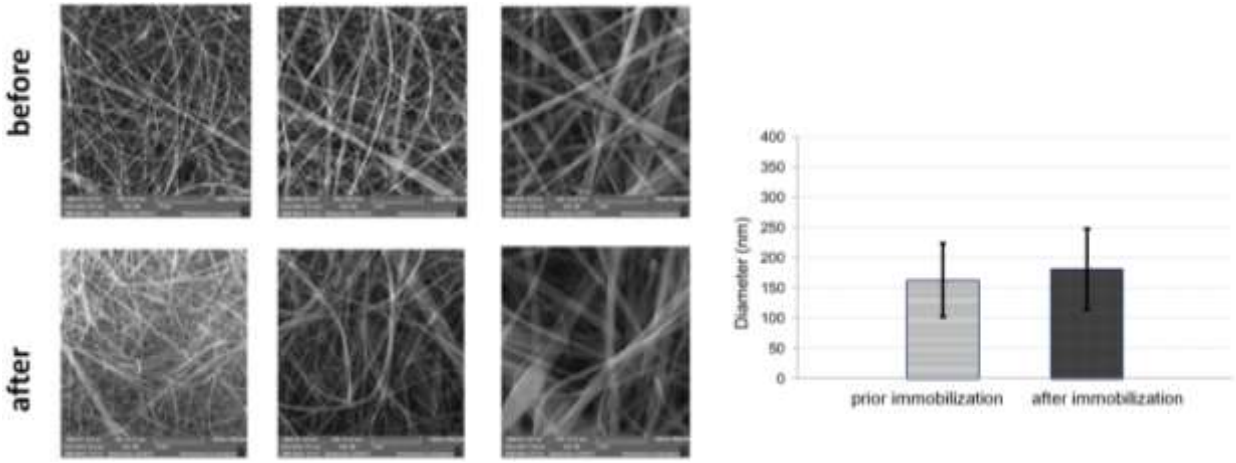

Fig. 1 SEM analysis of silica nanofibers prior and after immobilization of trypsin (magnification of 5.0, 10.0 and 25.0 kx, from the left); evaluation of mean fiber diameter ± SD prior and after the procedure. No significant difference proven (ANOVA, $p>0.05$).

Subsequently, we verified the storage and the operational stability of the immobilized enzyme as important factors which ensure its proper and efficient application. Operational stability of covalently bound trypsin on silica nanofibers was determined (Fig. 2). The aim of operational stability determination was to find out possible changes in immobilized trypsin activity after repeated use. Enzyme activity was measured in 6 consecutive assays during one day. Temporally decrease of trypsin activity observed is probably caused by reconstitution of enzyme molecule conformation after immobilization and also by gradual adaptation to the microenvironment. The last two enzyme activity determinations display the original activity values measured after immobilization and correspond to optimal conformational arrangement of enzyme molecules. The results demonstrate that bioactive system consisting of silica nanofiber layer with bound trypsin can be used repeatedly without any significant loss of biocatalyst activity.

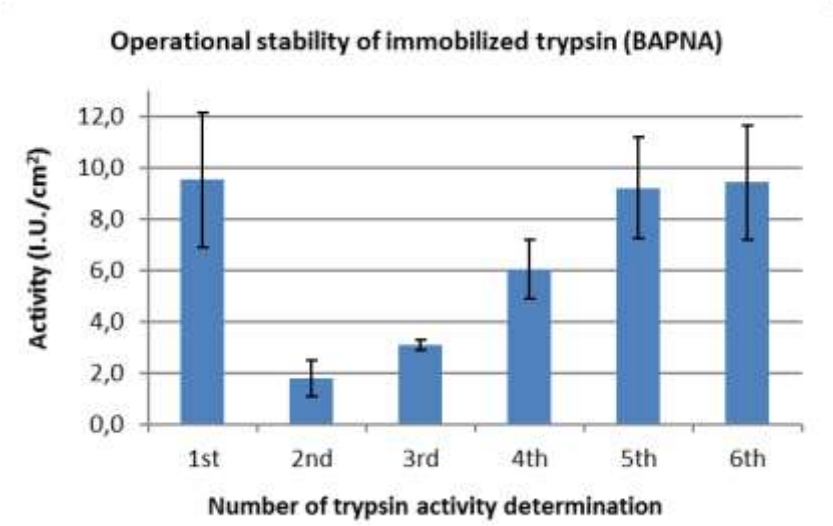

Fig. 2 Operational stability of trypsin immobilized on silica nanofibers (enzyme activity was determined using low-molecular weight substrate BAPNA in consecutive assays during one day).

Storage stability of trypsin immobilized on silica nanofibers was studied (Fig. 3). The enzyme activity (BAPNA) was determined in each triplicate after several storage days in 0.1M phosphate buffer (pH 7.3) at 4°C. The results indicate that during one week, trypsin activity remained unchanged in comparison with the activity value obtained immediately after immobilization. Temporary increase in trypsin activity was observed. This effect could be due to restoration of the biocatalyst proper conformation.The results also show that trypsin immobilized on silica nanofibers is quite stable at the prescribed storage conditions.

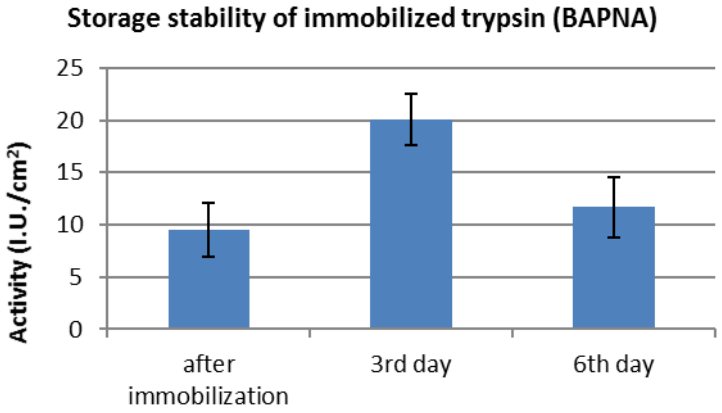

Fig. 3 Storage stability of immobilized trypsin (0.1M phosphate buffer, pH 7.3; 4 °C; enzyme activity was determined using low-molecular weight substrate BAPNA).

Normally the immobilized trypsin is localized in a special microenvironment provided by the support material and could therefore be shielded from microbial attack and oxidation thus enhancing stability against microbial degradation. Trypsin immobilization also brings benefit in preventing autolysis thus making immobilized molecule relatively more stable compared to its free form while conserving the same storage conditions [19].

CONCLUSION

The newly developed silica nanofibers were applied as a support for enzyme immobilization, namely the trypsin. Unique properties of this type of inorganic carrier as decreased water and protein adsorption, low immunogenicity or diminished swelling capacity in contact with water ensuring preservation of the pore size distribution in nanofibers structure, offer its advantageous use in various applications, especially in biomedicine. Considering the proteolytic activity of the selected biocatalyst and its ability to digest among others the necrotic tissue, we proposed here a prospective instrument for so discussed problem as is the healing of chronic wounds.

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