PRIMARY EVALUATION OF PCL/PEI NANOFIBRES IN TERMS OF THE SURFACE FUNCTIONALITY FOR BIOMOLECULES ATTACHMENT

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Abstract

Nanofibrous matrices represent an attractive material for biomedical applications - mainly due to their high specific surface and small pore size, which make them interesting material for drug delivery matrixes construction. Besides the morphological characteristics, the surface functionality strongly affects the drug-matrix interaction, drug conjugation and drug release kinetics. Implication of primary amino (-NH_2) groups to the surface of nanofibres can improve their performance and enable conjugation of drug via covalent binding and/or electrostatic interactions. The aim of this study was to evaluate applicability a nanofibrous material based on blend of two biocompatible and biodegradable polymers - poly-Ɛ-caprolactone (PCL) and polyethylenimine (PEI) produced by needleless electrospinning. The electrospun matrices were evaluated in terms of morphology, functional groups availability and short term stability. Moreover, biocompatibility to the 3T3 fibroblastic cell line was verified in direct contact. Binding capacity estimation was performed on a model molecule (BSA) in order to demonstrate binding capacity of the system and its applicability for protein immobilization for wound management.

Keywords: Nanofibres, polycaprolactone (PCL), polyethylenimine (PEI)

1. INTRODUCTION

During the last two decades, electrospun fibers have been shown to have an important potential in biomedical application. Their perspectives in improving human health originate from high specific surface area combined with high porosity and small pore sizes. While application as a barrier system have found use as skin covers in wound healing, their specific 3D structure gained profit as scaffolds in tissue engineering. Moreover, their high specific surface provides benefit in incorporating and/or carrying higher amount of biomolecules which can serve for specific drug loading and/or targeting, and enable the controlled delivery and precise release of the drug amount wanted in the site of interest defined. Since the synthesized biomaterials enter in contact with the complex system of living matter, they need to have perfect surface properties. The surface treatment of such materials enter the game here to provide the required clinical performance and to allow the enhanced adaptability to the physiological surroundings [1]. The new-generation nano-biomaterials are therefore advanced in surface physico-chemical modification comprising (and often combining) adsorption, covalent binding, ion implantation, deposition and conversion [2], techniques known as biofunctionalization (i.e. [3, 4]) which can regulate biological responses [5]. A number of functionalisations rely on the existence of surface-accessible functional groups available for further modifications. Functional groups such as amine, imine, carboxylic etc. provide the nanomaterial with specific properties by making its surface active [6] and available for furher immobilization of molecules [7, 8, 9]. Polyethylenimine (PEI), for example, which contains primary, secondary, and tertiary amine groups, is one of the common cationic biocompatible polymers providing such activity to the nanoscaled surfaces [10]. The surface treatment with PEI has also been shown to be antibacterial in certain conditions [11, 12] which can be a benefit for the most part of the so-modified bio-nanomaterials with clinical application. This bio-nanomaterial can be poly-Ɛ-caprolactone (PCL) which has been recently proposed and tested as one of the most promising polymers for advanced biomedical applications [13]. It is mainly due

to its excellent biocompatibility, slow degradation, low prices and reproducibility, nontoxicity and excellent mechanical properties [14]. Since the hydrophobicity of PCL is undesirable for most clinical applications, this polymer is proposed to prepare carriers in particular in the form of composites with a hydrophilic polymer [15]. In addition to the aforementioned benefits, the already mentioned PEI is a suitable polymer capable of providing the nanomaterial this increase in hydrophilicity [16].

In our work, we have prepared the nanofibrous composite layer based on two biocompatible and biodegradable polymers (PCL and PEI) where PEI as the surface functionalizing molecule is embedded directly into the system, thus eliminating the need for additional surface treatment of the carrier. We have therefore replaced the two-step preparation of a functionalized system with a time-less one-step preparation. Compared to the PCL/PEI nanofibrous composites formed by needle electrode [17], application of the needleless electrospinning system leads to formation of homogenous nanofibrous layer and can be easily up scaled to mass production. The newly prepared composite was evaluated in terms of morphology, chemical composition, functional groups availability and short term stability. Verification of the biocompatibility of the system to the 3T3 fibroblasts as well as its binding capacity towards BSA as model molecule were also evaluated.

2. MATERIALS AND METHODS

2.1. Electrospinning process

The polycaprolactone (PCL, Mn 45.000) and polyethylenimine (PEI, Mw 25.000, branched) were obtained from Sigma-Aldrich, Co. Solvents used for the electrospinning process were obtained from Ing. Petr Švec - Penta, s.r.o. Both polymers were dissolved in a solvent system based on chloroform as a solvent common to both. The final spinning solution composed of PCL:PEI in ratio 85:15 was electrospun using a needleless electrospinning (wire electrode) under high voltage (60 kV) on distance of 150 mm. The electrospun nanofibres were consequently characterized in terms of morphology, surface functionality and biocompatibility as a relevant parameters for further application.

2.2. Morphological characterization

Morphology of the electrospun nanofibres was characterized by electron microscopy examination. Nanofibres prior and after the solvent residues extraction (washing in UPW, 48 hrs) were dried, sputter coated (Au, 5 nm) and analyzed under gradual magnification (up to 25.000x) on the SEM Vega 3 Tescan.

2.3. The surface functionality analysis

Content of the functional amino groups available on the surface of the nanofibres was estimated spectrophotometrically due to specific reaction based on methyl orange (MO, Sigma-Aldrich, Co.) binding to primary amino groups. For the analysis, samples of nanofibres prior and after solvent residues extraction (24 and 48 hrs) were incubated in the MO solution (0.05% MO in 0.01M natrium dihydrogen phosphate solution) for one hour. Subsequently, the unbounded dye was washed out. Afterwards, the MO was released by rinse in 0.1M sodium carbonate solution. Quantum on the available functional groups was calculated based on the bounded MO concentration by reading on 465 nm. The experiment was performed in six repeats per sample.

2.4. Immobilization of a model molecule

Performance of the composite PCL:PEI nanofibres for the potential immobilization procedure was evaluated using model molecule - the bovine serum albumine protein (BSA,≥96%, Sigma-Aldrich, Co.). The immobilization was performed using carbodiimide crosllinking chemistry. The ability of the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich, Co.) to covalently conjugate proteins was supported by addition of N-hydroxysuccinimide (NHS, Sigma-Aldrich, Co.) to the reaction mixture. Effect of the pH on the reaction performance was studied while the BSA concentration in the reaction mixture was sustained at 0.1 %.

The amount of the immobilized protein was estimated using Pierce™ BCA Protein Assay Kit (Thermo Scientific) based on bicinchoninic acid colorimetric reaction. The assay was performed according to the manufacturer´s instructions on microplate. Color product of the reaction was quantified reading at 562 nm (Synergy™ HTX, BioTek) and the protein amount was calculated through blank-corrected calibration curve constructed for BSA as standard. Test was performed in six replicates for each sample.

2.5. Biocompatibility evaluation

Potential cytotoxic effect of the PCL:PEI nanofibres was evaluated *in vitro* by test in direct contact. The test was performed in compliance with standard ISO 10993-5: 2009. Before exposure to the tested samples, 3T3 fibroblast cell line (A31 clone) was pre-cultured under standard conditions for 24 hours. Solvent residues were extracted from nanofibres for 24 hours prior the test. Tested samples were compared to cell control (CC), positive (PC, PM-A, Hatano Research Institute, FDSC, Japan) and negative (NC, RM-C, Hatano Research Institute, FDSC, Japan) control. Cell viability was evaluated after 24 hours of exposure in complete culture media (DMEM, 5% FBS, 5% NBCS) via standard mitochondrial oxidoreductases metabolic activity test. Amount of produced MTT formazan was determined spectrophotometrically by reading at 570nm (background substracted at 650nm). Testing of each sample was performed in triplicate for each sample.

3. RESULTS

3.1. Morphological characterization

The electron microscopy evaluation of the electrospun PCL:PEI nanofibres confirmed fibrous morphology to be obtained by the electrospinning technique and sustained after the extraction procedure. As shown below (**Figure 1b**), the extraction procedure did not lead to mayor swelling of fibers neither closing of the inter-fibrous pores and therefore decrease on the porosity of the nanofibrous sheet. This results indicate that the PCL:PEI nanofibres have potential to serve as a matrix for immobilization and application of biotechnologically relevant molecules in moist environment without loss of their morphological characteristics - namely high specific surface and porosity. The morphological evaluation also revealed formation of the pores on the fibers´ surface after the extraction procedure. This effect might actually lead to additional improvement in specific surface available.

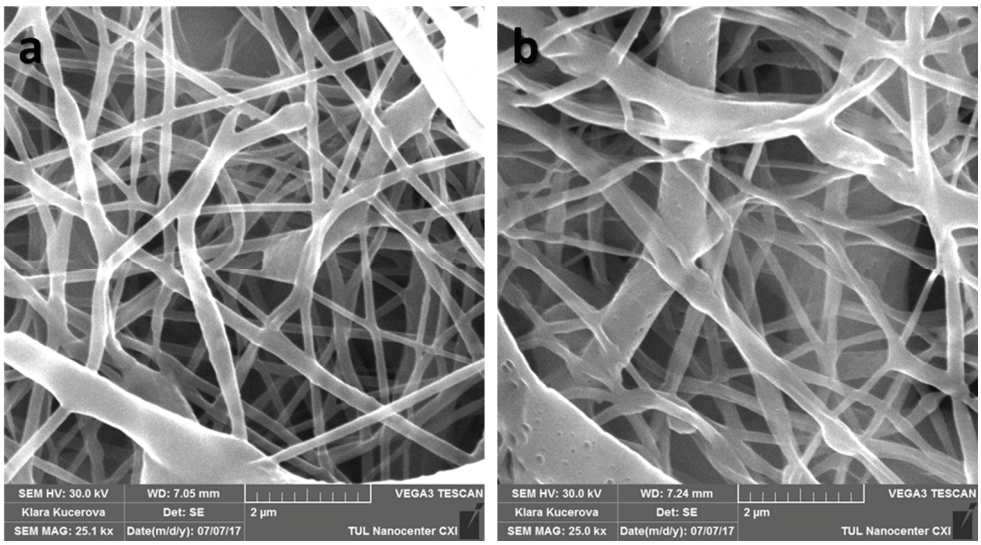

Figure 1 SEM images of the PCL:PEI nanofibres (a) prior the solvent extraction and (b) after the solvent extraction. Mag. 25.000, scale bar = 2 µm

3.2. The surface functionality analysis

The surface functionality analysis was performed in order to evaluate a potential loss of amino groups available on the surface after the extraction procedure due to the PEI solubility in water. This assumption was not confirmed and the results shown in **Figure 2** reveal increase in number of the available functional groups uncovered by the solvent extraction procedure. Meanwhile, the PCL:PEI surface prior the extraction contained 58.6 ± 9.2 nmol of available NH_2 groups per mg of nanofibres (NFs), their number increased to 119.5 ± 17.2 nmol NH_2/mg NFs after 24 hours of extraction and up to 165.9 ± 26.5 nmol NH_2/mg NFs after another 24 hours (48 hours of extraction in total).

This effect could be explained by slight swelling and changes in the morphology of the nanofibres during the extraction process, which might lead to increase of specific surface and uncovering of the PEI chains. The unfulfilled decrease in number of functional groups - e.g. not washing the PEI component away, suggests sufficient entanglement of the two types of polymer chains or formation of a semi-IPN due to electrostatic interactions. This assumption must be confirmed by further investigation.

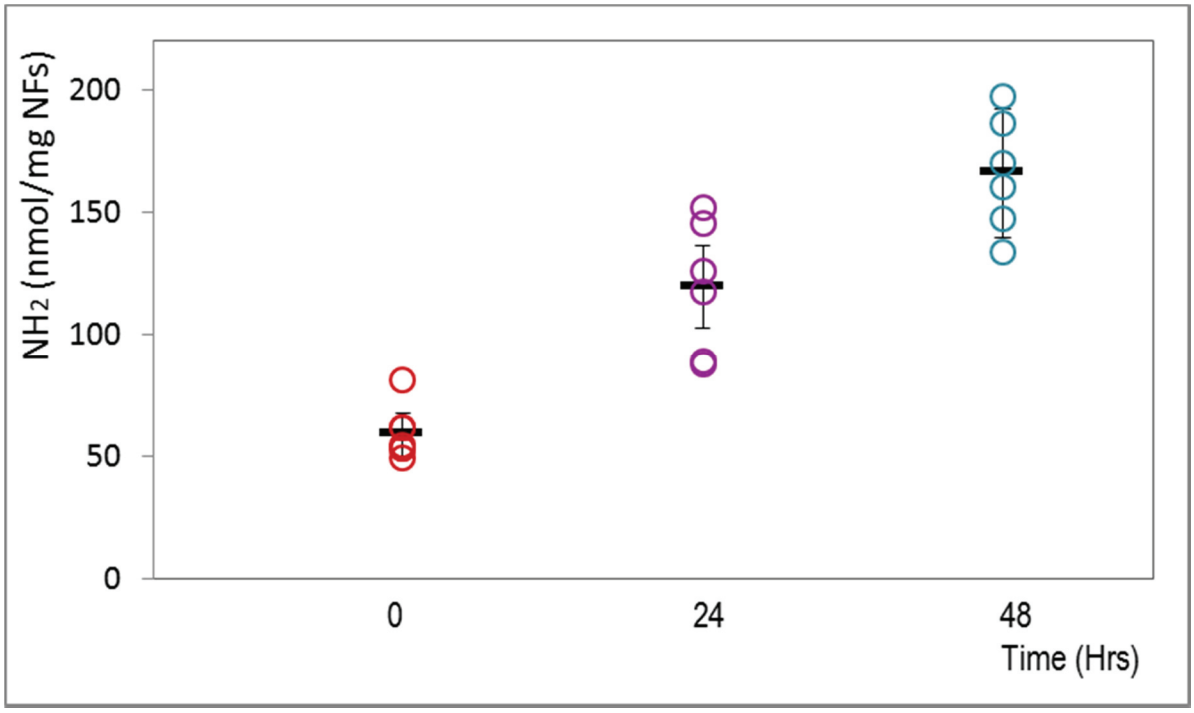

Figure 2 Graphical representation of the available amino groups results and their correlation to extraction time. Results of a single measurements (○) are completed with MEAN ± S.D.(—)

3.3. Immobilization of the model molecule

Evaluation of the availability of the functional groups was extended by a model drug conjugation and quantification. The chosen model drug - bovine serum albumin (BSA) was immobilized to the surface using EDC/NHS conjugation. Effect of the reaction pH was evaluated cursorily in order to map the optimal reaction conditions as pH limitations are placed on many biomolecules and limit their applicability for conjugation chemistry requiring acidic conditions. Results of the BSA immobilization (**Figure 3**) confirmed the requirement of the acidic pH for the performance of EDC/NHS coupling reaction. The highest amount of protein (141.3 ± 23.7 µg BSA/ mg NFs) was immobilized under pH of 6. We expect this result to be a synergic effect of the carbodiimide method requirements and the BSA isoelectric point. Due to this observation, necessary modifications of the conjugation conditions are expected for each molecule immobilized by this conjugation method.

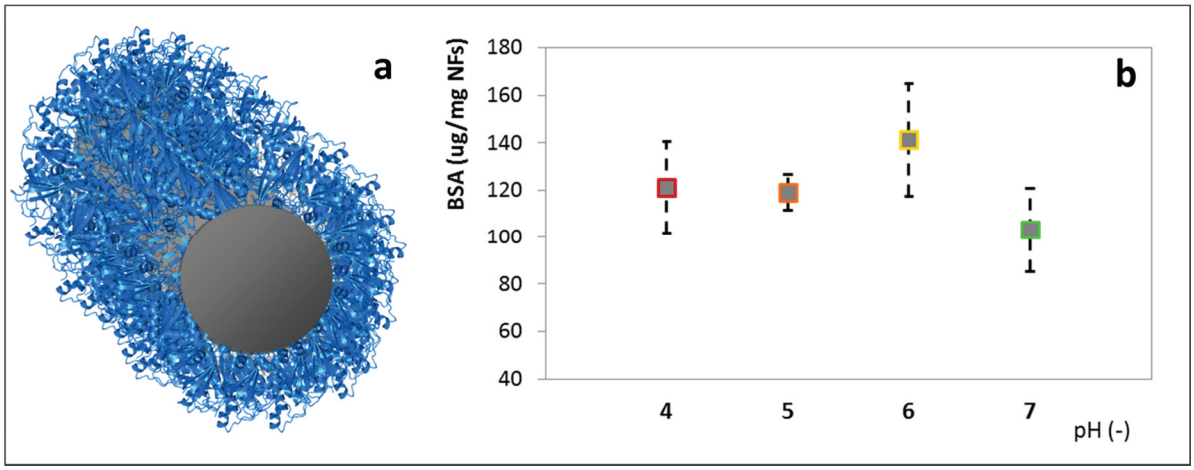

Figure 3 a. Illustrative representation of the BSA protein bonded to the surface of nanofibres. b. Results of quantification of the model molecule immobilized to the surface of the nanofibres/effect of the reaction pH.

3.4. Biocompatibility evaluation

The *in vitro* evaluation proved biocompatibility of the PCL:PEI nanofibres. The viability of 3T3 fibroblast cells after exposure to the nanofibrous sheet exhibited viability of 91.8 ± 6.9% compared to the cell control (CC). The positive and negative controls were applied to evaluate reliability of the test. The positive control (PC) application led to cell viability of 39.4 ± 11.7% and the negative control reached viability of 87.4 ± 3.3%. The results of the biocompatibility evaluation are displayed below (**Figure 4**). The level of viability exceeded the level of 80% of the CC cell number and due to this result the PCL:PEI nanofibres can be considered as biocompatible material.

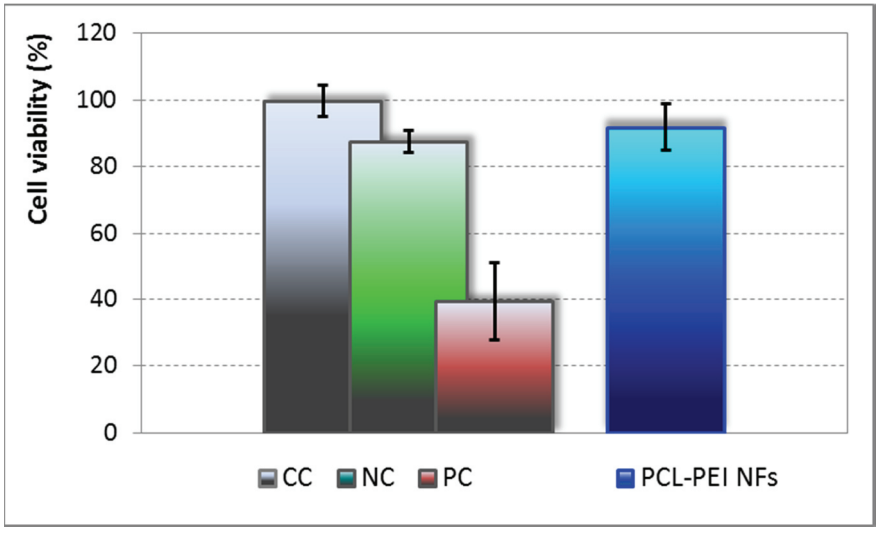

Figure 4 Results of the 3T3 cells viability after exposure to the tested samples. CC - cell control, NC - negative control, PC - positive control.

4. CONCLUSION

The PCL:PEI novel nanofibres were successfully electrospun via a needleless electrospinning procedure. Fibrous morphology in submicron scale was confirmed by SEM examination. Presence and sustainability of free amino groups on the surface was confirmed. Their increasing number caused by solvent extraction can be attributed to the slight changes in morphology of the fibers. Availability of the functional groups for covalent

conjugation of relevant molecules was demonstrated on a model BSA protein. The applicability of the system in biomedical is supported also by results of biocompatibility testing as the cell viability of the 3T3 cells exposed to the material reached 91.8 ± 6.9% and therefore the PCL:PEI nanofibres can be declared as biocompatible.

ACKNOWLEDGEMENTS

Realization and presentation of the research reported in this paper was supported by the TAČR project TH02020786 and by the MPO Trio projects FV10605 and FV10054 realized at the Institute for Nanomaterials, Novel Technologies and Innovation of the Technical University of Liberec.

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