INCORPORATION NITRIC OXIDE DONORS INTO FIBROUS MATERIAL INTENDED FOR VASCULAR GRAFTS

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Abstract

Nitric Oxide (NO) is a very promising molecule suitable for applications in cardiovascular system. NO has many beneficial effects such as anti-thrombogenic and anti-inflammatory properties together with endothelial cell support. Presented work was focused on functionalization of electrospun biodegradable materials by nitric oxide donors for further usage as vascular grafts. Firstly, the optimization of biodegradable nanofibrous layers was carried out. Fibrous sheets were produced from the aliphatic polyester polycaprolactone (PCL) that was blended with nitric oxide donor S-nitrosoacetyl N-penicillamine (SNAPs) in various concentrations of 0.2 wt % and 0.4 wt%. Subsequently, prepared layers with and without NO donors were tested *in vitro* using endothelial cells and its effect was compared. Nitric oxide release from functionalized layers was detected by colorimetric Griess assay after 1, 2 and 3 days of incubation. Moreover, sterilization methods and their impact for NO release was monitored.

Keywords: Nitric oxide (NO), nanofibers, vascular grafts, Griess assay, scaffolds

1. INTRODUCTION

Nitric oxide (NO) is a very interesting molecule in the human body especially for cardiovascular system. Firstly, the molecule was described in 1980s. Later in 1992, nitric oxide was selected by scientific journal authors as the molecule of the year. In 1998, three scientists R. F. Furchgott, L. J. Ignarro and F. Murad were awarded the Nobel Prize in Physiology or Medicine for their input and explanation of the function of NO in living organisms. Nitric oxide is a gas under normal climatic conditions. NO is important biological molecule effecting a lot of cell types such as endothelial cells, smooth muscle cells, platelets and immune cells. Nitric oxide support endothelial cell proliferation in living organisms [1]. Appropriate NO release is beneficial for development of functional vascular grafts that usually lack endothelialization of the lumen, possess thrombogenic and/or inflammatory properties or led to intimal hyperplasia thickening due to the over proliferation of smooth muscle cells [2]. Incorporation of NO donors into the structure of vascular prosthesis will prevent the most common graft failure.

1.1. Griess assay

Griess assay is spectrophotometric method used for determination of nitric oxide, nitrates and nitrites (NOx) [1]. The method is based on the reaction of NOx with sulfanilamide (SULF) or sulfanilic acid (SA). Formed product react with N- (1-naphthyl) ethylene (NEDD) that yields in orage azo-dye absorbed at 496 nm when incubated in PBS or in purple colored dye absorbing at 540 nm after incubation in water. The absorbance of the solutions after incubation with functionalization materials was related to NO concentration based on calibration curve construction [3].

1.2. SNAPs Synthesis

S-nitrosoacetyl-N-penicillamine was synthesized by dissolving N-acetyl-DL-penicillamine in methanol by sonification. Then, sulfuric acid and hydrochloric acid were added for adjusting pH. Subsequently, the solution

was cooled with ice for one hour leading to a green-red color. Then, the solution was evaporated in a vacuum evaporator where methanol was removed. The final drying of the product, SNAPs, was carried out at room temperature for approximately two days.

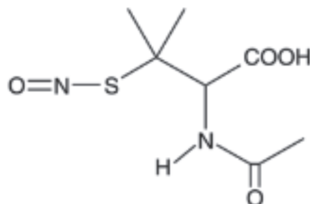


Figure 1 Chemical structure of SNAPs

1.3. Materials

Polycaprolactone (PCL, Sigma Aldrich, Mw 45000 g / mol) as a representative of aliphatic polyesters was electrospun using Nanospider™. The polymer was dissolved in solvent system composed of chloroform, acetic acid and ethanol in a ratio of 8/1/1 (v / v / v) in a final concentration of 16 wt %. Functionalization of the fibrous layer was done by blending of polymeric solution with various concentrations of NO donor. SNAPs were added to the electrospinning solution at concentrations of 0.2 and 0.4 wt % one hour before electrostatic spinning.

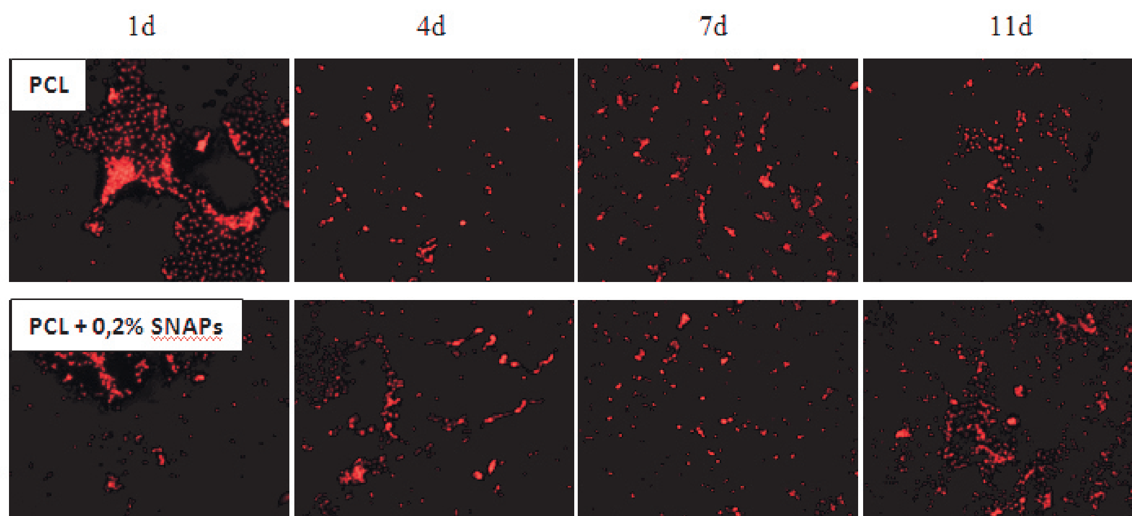
After electrospinning, the layers were examined by scanning electrone microscopy and the fiber diameter was measured using image analysis. Average fiber diameters ± standard deviations of resulting fibrous layers were:

a) PCL: 325.4 ± 113.02 nm
b) PCL + 0.2 % SNAPs: 306.1 ± 146.19 nm
c) PCL + 0.4 % SNAPs: 272.66 ± 111.94 nm

2. CULTIVATION ENDOTHELIAL CELLS

Nitric oxide is known to support endothelial cell proliferation. [2]. Prior to cell seeding, materials were sterilized by immersion in 70% ethanol and washed twice in PBS for 5 minutes. Produced materials (PCL, PCL+0.2% SNAPs, PCL+0.4% SNAPs) were incubated with human umbilical vein endothelial cells (HUVEC, Lonza) and analyzed by fluorescence and scanning electrone microscopy.

2.1. Fluorescence microscopy



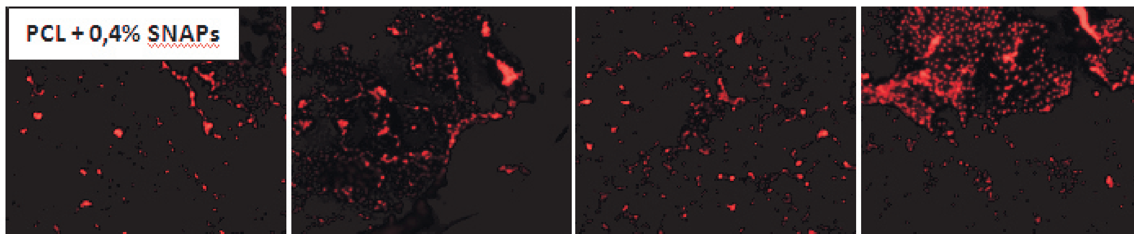


Figure 2 Images from fluorescence microscopy after dying of cell nuclei with propidium iodide for tested materials PCL (1st row), PCL + 0.2% SNAPS (2nd row) and PCL + 0.4% SNAPS (3rd row) after 1 day of cultivation (1st column), 4 days (2nd column), 7 days (3rd column) and 11 days (4th column)

Endothelial cells were fixed and stained with propidium iodide in red after 1, 4, 7 and 11 days of incubation. Scaffolds with seeded cells were captured using fluorescence microscope. The results are summarized in the following **Figure 2**. The number of cells did not show significant differences between tested materials, but even between individual days of incubation. Endothelial cells adhered to fibrous materials but further proliferation did not occur. In case of PCL+0.4% SNAPs, islands of endothelial cells were found.

2.2. SEM

Fixed cells cultured on fibrous materials were assessed using scanning electron microscopy as depicted in **Figure 3**. Cells adhered on the nanofibrous structure as seen from the first column of **Figure 3**. The images of PCL 0.2 and 0.4 % SNAPs in the seventh and eleventh day show a continuous layer of cell. These figures indicate that the content of SNAPs have a positive effect on endothelial cell growth. However, quantification of cells and further tests will have to be carried out in order to prove the statement.

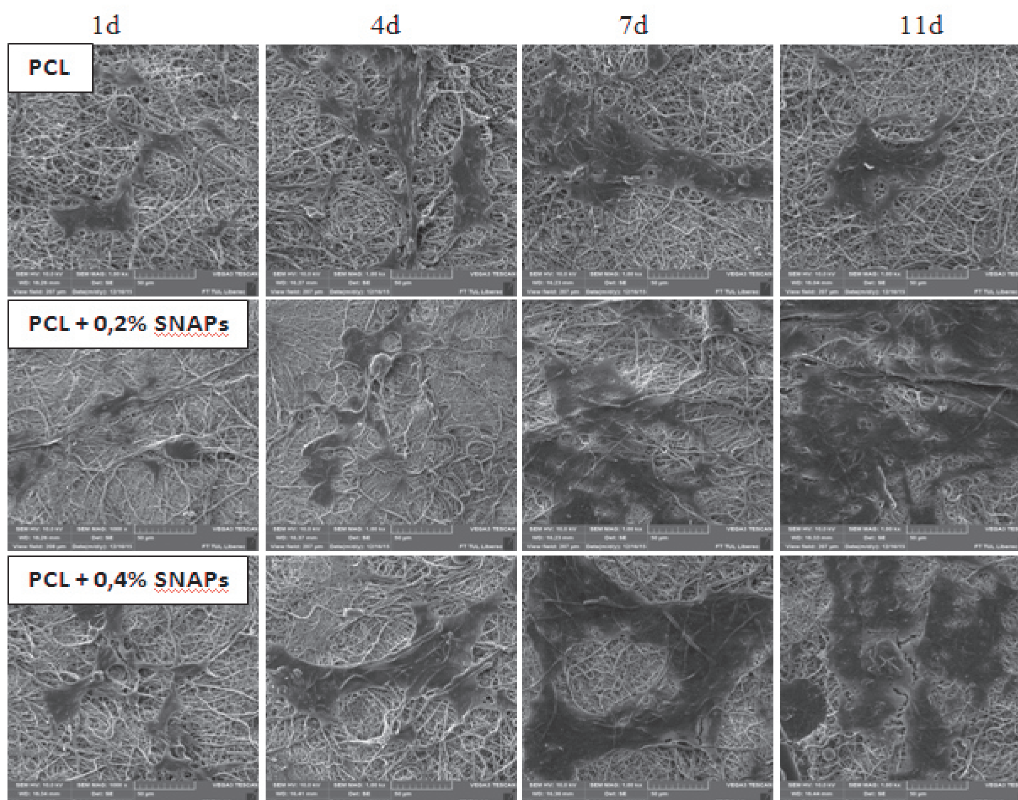


Figure 3 Images of scanning electron microscopy showing the endothelial cells cultured on PCL nanofiber (1st row), PCL + 0.2% SNAPS (2nd row) and PCL + 0.4% SNAPS (3rd row) after 1 day of culture (1st column), 4 days (2nd column), 7 days (3rd column) and 11 days (4th column)

2.3. Kinetics release of NO

NO release from prepared materials was measured after 1, 2 and 3 days of incubation in PBS. For each material, 50 mg was weighted and inserted into 15 ml tubes containing PBS / water. After certain incubation period, Griess assay in water and in PBS was carried out (n=5). The data of NO release after 1, 2 and 3 days were plotted cumulatively as mean ± standard deviation in **Figure 4**.

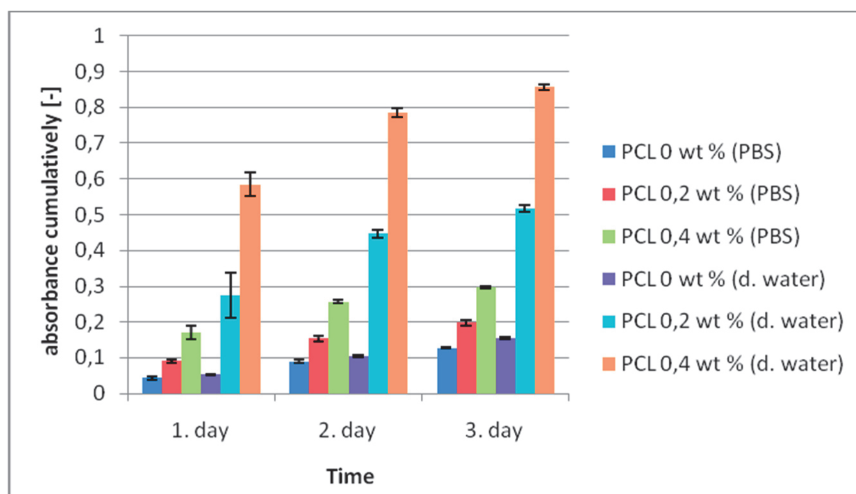


Figure 4 Cumulative NO release after 1, 2 and 3 days of electrospun materials incubation in PBS / water analyzed by Griess assay

Absorbance is presented cumulatively in the graph. The results indicate that the highest rate of NO release was within the first day. After the second and the third day, release of NO has slowed down. This may be caused by the release of NO from the fiber surface. Slow degradation rate of PCL do not allow NO donors to release further NO but the fibers could still contain SNAPs embedded in polymer.

2.4. Sterilization effect on NO release

Sterilization procedure can influence the content of final scaffold and its functionalization. Due to the unclear results of in vitro tests, sterilization technique was assessed for NO release. For comparison, ethanol sterilization was used in the same way as before *in vitro* experiment described above and ethylene oxide sterilization (Anprolene, 37°C). Similarly, 50 mg of each material underwent the sterilization procedure and further NO release was examined by Griess assay. The results are summarized in **Figure 5** indicating that sterilization technique strongly influences the NO release. Samples after immersion in ethanol and PBS and after ethylene oxide sterilization showed decreased NO release.

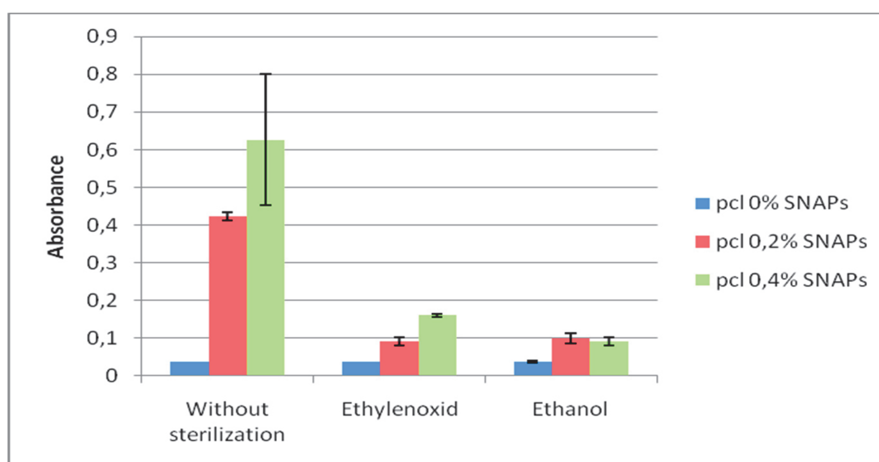


Figure 5 NO release from tested materials (PCL, PCL+0.2% SNAPs, PCL+0.4% SNAPs) in non-sterile form, after ethanol sterilization and ethylene oxide sterilization

3. CONCLUSION

Polycaprolactone was electrospun and functionalized with NO donors in order to improve vascular graft function. Various concentrations of SNAPs were blended with PCL (0.2% and 0.4%) leading to uniform fibrous structure capable of NO release for 3 days that was measured by Griess assay. *In vitro* tests were influenced by sterilization prior to cell seeding that decrease the level of NO available. Further it was confirmed that NO release is strongly influenced by sterilization method. When ethanol treatment or ethylene oxide was used, the NO release was much lower than in non-sterile samples. Therefore suitable sterilization method has to be thoroughly considered.

Acknowledgements

We are thankful to the Dpt. of Nonwovens and Nanofibrous materials, Faculty of Textile Engineering, Technical University of Liberec and to the student project „SGS-21092" and project "Nanofibrous Biodegradable Small-Diameter Vascular Bypass Graft" financed by the Ministry of Health (No. 15-29241A, 2015-2018).

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