Structure and properties of slow-resorbing nanofibers obtained by (co-axial) electrospinning as tissue scaffolds in regenerative medicine

Running title: bio-friendly coaxial electrospin

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Abbreviations: BF - Bright-Field; EDS - Energy Dispersive; DMF – Dimethylformamide; FTIR - Fourier Transform Infrared Spectroscopy; HAADF - High-Angle Annular Dark-Field; IFFT - Inverse Fast Fourier Transform; KBr - Potassium Bromide; N6 - Nylon 6; NHDF - Normal Human Dermal Fibroblasts; PCL- Polycaprolactone; PCL5/PCL10 - 5% polycaprolactone/10% polycaprolactone; PCL5/PCLAg - 5% polycaprolactone/polycaprolacone with silver nanoparticles; PCL10 - 10% polycaprolactone; PMMA - Poly(methyl methacrylate); PVA - Polyvinyl Alcohol; SEM - Scanning Electron Microscope; STEM - Scanning Transmission Electron Microscopy; TEM - Transmission Electron Microscopy; THF- Tetrahydrofuran; XRD - X-Ray Diffraction.
Summary

We investigated the structure and properties of PCL_{10} nanofiber, PCL_{5}/PCL_{10} core-shell type nanofibers, as well as PCL_{5}/PCL_{Ag} nanofibres prepared by electrospinning. For the production of the fibre variants, a 5-10% solution of polycaprolactone (M_w = 70000-90000), dissolved in a mixture of formic acid and acetic acid at a ratio of 70:30 m/m was used. In order to obtain fibres containing PCL_{Ag} 1% of silver nanoparticles was added. The electrospin was conducted using the above-described solutions at the electrostatic field. The subsequent bio-analysis shows that synthesis of core-shell nanofibers PCL_{5}/PCL_{10}, and the silver-doped variant nanofiber core shell PCL_{5}/PCL_{Ag} by using organic acids as solvents is a robust technique. Such way obtained nanofibres may then be used in regenerative medicine for extracellular scaffolds: (i) for controlled bone regeneration due to the long decay time of the PCL, (ii) and as carriers of drug delivery nanocapsules. Furthermore, the used solvents are significantly less toxic than the solvents for polycaprolactone currently commonly used in electrospin, like for example chloroform (CHCl_3), methanol (CH_3OH), dimethylformamide (C_3H_7NO) or tetrahyrofurna (C_4H_8O), hence the presented here electrospin technique may allow for the production of multilayer nanofibres more suitable for the use in medical field.

Keywords: polycaprolacotne; nanofibers; core-shell nanofibers; co-axial electrospinning; solution electrospinningen
1. Introduction

A process for preparing micro and nanofibres with the help of electrostatic field could be divided into electrospin from the raw material in solution (Fig. 1) and also from the melt. The initial properties of the polymeric material will dictate what kind of method for the preparation of the nanofibers. For natural polymers like chitosan, which do not melt under the influence of the temperature, hence electrospinning is chosen for the preparation of micro- and nanofibers [1, 2]. Melt electrospinning is chosen for the generation of nanofibres form the thermoplastic polymers such as polypropylene, which is difficult to dissolve but it melts at higher temperatures [3]. Polymeric materials exist, which could be converted into the nanofiber using electrospinning of the solution and also melt-electrospinning. As examples of these types of materials serve PVA [4], N6 [5], PMMA [6].

The most common method of preparation of nanofibres from the solution is the electrospinning process. Obtained in the process diameter of fibers, surface morphology, and regularity of shape depends on three main conditions: (i) environment (the temperature of gas in which electrospinning occurs, gas humidity, gas flow velocity), (ii) process conditions (i.e. the type of electrodes used, the flow rate of the solution, the distance between the electrodes, the rotational speed of the rotary collector), and (iii) the properties of the solution which is the sum of the properties of the polymeric material used, the properties of the solvent, and the properties of introduced additives. Each of these three conditions and the properties is directly or indirectly related with the others [9]. For example, the properties of the polymeric material will dictate the type of solvent, which is used to dissolve it [10]. Because most polymers are dielectrics, adding the solvent will decrease the conductivity of the solution. However, there are polymeric materials, such as chitosan, which when added to the solution, will increase the conductivity of the resulting solution. Converting the polymer solution into nanofibres is accompanied by evaporation of the used solvent.
A variation of the electrospinning process, called coaxial-electrospinning was employed in this paper (Fig. 1) [11]. Like during the standard electrospinning process, shortly after passing the nozzle, under optimal conditions, Taylor cone is created (double cone in coaxial electrospin). The shape and properties of Taylor's cones depend on the characteristic of the respected polymer-forming solutions. At the contact-zone both solutions interact, and the nature of this interaction is formative for the coaxial electrospinning process. The coating solution is stretched by electrostatic field within the zone of straightforward flow (according to the Faraday law, the charge accumulates on the outer wall of a structure). The flow of the core-forming solution is also stretched due to the interactions and friction imposed on it by the coating solution. The shaping (morphology) of nanofibers of the core-shell polymer-type is also strongly influenced by the evaporation rate of solvents used in the process. If the core-forming material is dissolved with a solvent with much higher evaporation rate than the solvent in the coating polymer, within the core a thin layer (wall) will form, that will cause the solvent to diffuse slowly. In effect, an under-pressure is created within the core, which will cause the collapse of the core, and results in a strip/ribbon shape of the nanofibre.

Coaxial electrospinning technology is significantly more difficult than the single-material electrospinning, however it enables the preparation of fibers having much broader applications. It allows for example, for obtaining a hollow fibers [12, 13], core-shell type fibers [14, 15], encapsulating other compounds and biologic materials [16], encapsulation of macromolecular materials such as i.e. DNA, preparation of composite fibers [17], combination of polymeric materials with different properties [18], or production of tissue scaffolding with a desired disintegration time [19]. The purpose of the following study was to prepare a core-shell nanofibers using a combination of two organic acids as solvents for polycaprolactone. Organic acids at trace-quantities are in general, better tolerated by living tissues, than organic solvents commonly used for electrospinning.
2. Materials and methods

2.1 Materials

For the preparation of a biodegradable composite fiber we used PCL polycaprolactone having a molecular weight $M_w = 70000-90000$, Sigma Aldrich. We used organic solvents: formic acid (99.9% purity), and acetic acid (also 99.9% purity) purchased from Sigma Aldrich. Silver nanoparticles with a diameter of 20-30nm, and the purity of 95.95% were from Skyspring Nanomaterials.

2.2 Dissolving the polymers

For the preparation of nanofibres applied:

a) a mixture of formic acid and acetic acid in a ratio of 70:30 m/m was prepared, to which the silver nanoparticles were introduced in an amount of 1%, while using a ultrasonic homogenizer for 30 min. Next, to the solution polycaprolactone ($M_w = 70000-90000$) was added, and dissolved, to give a 10% solution of PCL$_{Ag}$

b) a mixture of formic acid and acetic acid in a ratio of 70:30 m/m was prepared, to which the granules of polycaprolactone ($M_w = 70000-90000$) was added while stirring with the magnetic stirrer, until the 10% solution of PCL was obtained (PCL$_{10}$). Following a similar approach, also the PCL$_{5}$ solution was prepared

c) mixture of formic acid and acetic acid in a ratio of 70:30 m/m was prepared, to which the granules of polycaprolactone ($M_w = 70000-90000$) was added while stirring with the magnetic stirrer, until the 5% solution of PCL was obtained (PCL$_{5}$)

2.3 Electrospinning
The PCL_5-, PCL_10-, and PCL/Ag-solutions (prepared as described above) were used. For the preparation of solid (one layer) nanofibres the PCL_10 was used. The PCL_10 solution was combined with PCL_5 solution to obtain a core-shell nanofibers PCL_5/PCL_10. PCL_5 solution was combined with the PCL/Ag solution to prepare the PCL_5/PCL/Ag nanofibres. The electrospin-parameters were as follow: the flow range 0.05-1.5 ml/h, the voltage range 0.8-1.2 kV/cm, collector (negative electrode): SS Flat Plate (400mm x 400 mm), single nozzle injector (positive electrode) outer needle 0.9 mm, co-axial injector (positive electrode) inner needle 0.6 mm, outer needle 1.4 mm, humidity: 32% +/- 4%, temperature: 23°C +/- 2°C. The electrospin was done using Coaxial Electrospinning & Electrospray 550 from Yflow.

2.3 Testing of the obtained nanofibers

FTIR

The evaluation of the structure of polycaprolactone was performed using FTIR infrared spectroscopy. The technique is used to determine the presence of functional groups within PCL. For visualization purpose, the sample was combined with KBr powder in a proportion 0.1mg of fibers to 3g KBr, compressed to form pellets and then placed in a desiccator with a moisture binder (silica gel) for 30 min at 40°C. After drying, the samples were placed in the autosampler of the device, and were scanned 128 times with a resolution of 4 cm^{-1} in wavelength range between 400 - 4000 cm^{-1}.

XRD

X-ray diffraction techniques was used to determine the structure of silver nanoparticles in PCL_5/PCL/Ag nanofibres. The X-ray diffraction measurements for selected samples were performed at ambient temperature using a Rigaku MiniFlex 600 Diffractometer (Rigaku.
Corporation, Tokyo, Japan) with Cu Kα radiation (λ = 1.5406 Å), at tube voltage of 40 kV and a current of 15 mA using a D/teX Ultra silicon strip detector.

**SEM & TEM**

The topography of the obtained nanofibres were analyzed by using scanning electron microscope Zeiss Supra at different settings of the acceleration voltage and magnification ranges, selected for optimal observation of samples. Samples of the tested nanofibres (PCL$_{10}$, PCL$_5$/PCL$_{10}$, PCL$_5$/PCL$_{Ag}$) were applied directly onto the surface of copper mesh and subjected to structural analysis using a high resolution transmission electron microscope TEM at an accelerating voltage of 300 kV and modes of the Fourier transformation FFT inverse Fourier transformation IFFT, using a bright-field detector BF, a high resolution wide angle dark field detector HAADF, STEM scan mode transmission, and a standard EDS detector.

**Cell viability tests**

The biocompatibility (potential toxicity) of the generated nanofibres was assessed by MTT assay using NHDF cells obtained from Professor G. Kratz, [20]. The procedures were performed similarly as described previously [21]. The cells were cultured in DMEM-F12 (SIGMA) supplemented with 10% FBS fetal calf serum solution (SIGMA), this sterile and standard conditions (37°C, 60% humidity, 5% CO$_2$), while being kept in logarythmic phase. For testing, cells were trypsinized, and plated on the test composites placed in 96-well plates (final density suspend 2x10$^4$ cells/well). Following 72 h incubation, MTT-reagent (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added at concentration 0.5 mg/ml. After further 4 h incubation, the MTT solution was removed and the formazan product was dissolved in dimethyl sulfoxide. The absorbance was measured at 550 nm with a VICTORTM X Series Multiple Plate Reader. The principle of the MTT reaction is
shown in Fig. 2.

**Confocal microscopy**

Biological studies were conducted on cell line NHDF (Normal Human Dermal Fibroblasts). The composite material with diameter equal to 2 cm was UV-sterilized and then it was placed into a cell culture incubator. One each sample, a cell suspension volume of 200 µl (~2x 10^5 cells) was applied, and then placed into incubator for 1,5 h at a temperature of 37°C. After 1,5 h in the incubator 3 ml of culture medium was added to each sample. The samples were then incubated for 96 h at 37°C. The tests were performed on three samples, for each fibre type. In order to determine the cells presence and their adhesion to the surface of the nanofibers the samples were removed from the incubators, after 96h of culture, then placed onto a glass slides and fixed with ethyl alcohol. After fixation the samples were stained with 40 µg/ml of propidim iodide to visualize the DNA in the cells. The excess of propidim iodide was removed by washing the samples with distilled water. Later each sample surface was covered with glycerine and then covered with a coverslip. The prepared samples were inspected by confocal microscopy (600 nm wavelength).
3. Results

The polycaprolactone nanofibers obtained by electrospin from its 10% solution in a mixture of formic acid and acetic acid were analyzed by the spectrometer FTIR (Fig. 3). The analysis confirmed the presence of: (i) stretching vibrations of C = O for the wave 1720 cm\(^{-1}\), (ii) symmetrical stretching vibration of C-H\(_2\) of wave 2866 cm\(^{-1}\), (iii) asymmetrical stretching vibration C-H\(_2\) wave 2943 cm\(^{-1}\), (iv) the stretching vibration of C-O and C-C for the wave 1294 cm\(^{-1}\), and (v) the stretching vibration of C-O and C-C to wave 1163 cm\(^{-1}\), characteristic for polycaprolactone. Using scanning electron microscopy, SEM (Fig. 4A), and TEM transmission electron microscopy (Fig. 5A) confirmed the presence of polycaprolactone nanofibers in the test samples. The use of a combination of formic acid with good electrical conductivity and acetic acid with low electrical conductivity in a proportion of 70:30 m/m as a starting PCL solvents, proved to be a successful solution allowing for stabilization of the electrospinning process of nanofibers. It also allowed for obtaining a core-shell nanofibers with the diameter of less than 100nm. (Fig. 5B and Fig. 6AB). The core and shell components are clearly visible both due to differential density of the respective forming materials, and also due to their different thickness. While the same material (PLC) was used for both the core and the shell formation, its starting concentrations were different. The differences in initial concentrations of used PLC solutions effected in different evaporation rates and effected in the final differential density of formed core and shell.

SEM-based investigation of obtained nanofibres (Fig. 4B) made it possible to determine the grain structure of the used silver nanoparticles. The presence of silver was also confirmed by studies using EDS diffractometer (Fig. 6). The data showed the presence of the plane (200) characteristic of silver. In order of further confirm the components of these nanoparticles a high-resolution TEM image from the selected area of figure 7 was shown in
figure 7IFFT and Fig. 7FFT. The lattice fringe of 0.205 nm correspond to the (200) plane of cubic silver.

Polycaprolactone-ray diffraction studies (Fig. 3) enabled, in turn, to determine the occurrence of two strong peaks 2Ø for 22°C and 24°C, which correspond to the reflexes (110) and (200) typical polycaprolacton.

Studies using TEM in bright-field (BF) mode, confirmed the presence of nanofibers of solid-type PCL10 (Fig. 5A), coaxial nanofibers PCL5/PCL10 (Fig. 5B), and PCL5/PCLAg (Fig. 6A TEM BF-mode and Fig. 6B TEM STEM-HAADF-mode).

PCL10 fibers, coaxial PCL5/PCL10-, and PCL5/PCLAg fibres, all are characterized by regularity in diameter and lack of surface defects such as so-called beads. Hence, this shows that the combination of solvents used, and the chosen molecular weight of the polymer, allow for reliable production of nanofibers, through a very stable electrospinning process.

Using TEM, we estimated the diameter of the PCL10 nanofibers to be 98 ± 5 nm, the diameter of the obtained coaxial filaments PCL5/PCL10 to be 93 ± 5 nm, whereas the diameter of the silver-nanoparticle-doped coaxial nanofibers PCL5/PCLAg to be 86 ± 5 nm. Interestingly, we have observed the decrease in the diameter of the coaxial nanofiber PCL5/PCL10 as compared to solid nanofibers PCL10, and a further decline in fiber diameter for the silver-nanoparticle doped coaxial nanofibres PCL5/PCLAg as compared to coaxial nanofibers PCL5/PCL10 that do not contain the silver nanoparticles.

The diameter of the core of the coaxial nanofibers PCL5/PCL10, is 15 ± 3 nm, and it represents 17-19% of the total diameter of the fiber. Whereas for the silver-nanoparticle doped nanofibres PCL5/PCLAg, the diameter is 12 ± 3 nm, thus representing 14-16% of the total diameter of the fiber. Thus, the thinning of the silver-doped nanofibres was mainly due to the thinner core produced by the electrospinning process. Importantly, in both types of coaxial nanofibres, the cores occupy central part of the fiber. This shows that the
electrospinning produced high-quality nanofibres (the thickness of the coating evenly distributed around the core).

The biocompatibility (potential cytotoxicity) of the produced coaxial nanofibres was assessed using propidium iodide staining of cell nuclei and subsequent observation under confocal microscopy (assessment of shrunken apoptotic nuclei, Fig. 8A), and by MTT assay (detection of mitochondrial oxidation, Fig. 8B). The data show that both types of nanofibers (PCL$_5$/PCL$_{10}$ and PCL$_5$/PCL$_{Ag}$) exhibit good biocompatibility, as assessed using NHDF cells. Surfaces of the tested coaxial nanofiber PCL$_5$/PCL$_{10}$ and PCL$_5$/PCL$_{Ag}$ are not toxic to the cells and allow them for attachment and metabolic activity. While further more advanced tests are necessary, the preliminary data indicate that the structure of the obtained filaments supports NHDF cell proliferation, thus these materials should be subjected to *in vivo* assays to assess their potential use as a tissue scaffold replacements.

4.0 Discussion

Our study have proven the possibility of obtaining core-shell nanofibers in an electrostatic field with a 10% solution of the polymer dissolved in a mixture of formic acid and acetic acid in a ratio of 70:30 m/m. Biomaterials which are produced to substitute natural tissue scaffolds should meet certain criteria, among others, lack of toxicity, biocompatibility, sufficient strength, the optimal porosity and surface available for cell adhesion [22]. Compliance with these criteria is a necessary condition for the biomaterial to functionally restore the natural structure of the tissue.

The lack of toxicity of the electrospinned scaffolds depends on the properties of the starting materials from which it was made, and also to the lesser degree, on the used solvents (traces of solvents usually are difficult to be removed form the biomaterial), it also depends on possible contaminants, i.e. metal ions that may be toxic [23]. The literature frequently
describes DMF (dimethylformamide), THF (tetrahydrofuran), chloroform, or methanol as solvents used for polycaprolactone tested in this work [8, 9, 12]. These organic solvents are highly toxic to cells, and their residual amounts may leak from the implanted into the body biomaterial. This issue is typically ignored by the authors when discussing biocompatibility, and lack of toxicity of the electrospun biomaterials, mostly because such assessments are based on a short \textit{in vitro} studies, typically lasting a few days. Under such conditions the residual toxicity of the used organic solvents would unlikely be revealed. Such \textit{in vitro} studies will unlikely reveal residual toxicity of organic solvents, because the cells are in contact only with surface of the tested fibres, that usually would be solvent free (evaporated), and the tested material is immersed in large quantities of cell medium which further minimizes solvents’ potential toxicity. Upon implantation, the conditions differ, because as polycaprolactone degrades (complete degradation of polycaprolactone may take up to two years), it releases the residual organic solvents. Hence the \textit{in vitro} studies are unable unequivocally state whether the material throughout the period of degradation would be a nontoxic to-, and biocompatible with the surrounding cells because trapped in the structure solvents such as chloroform, DMF, or others, may be released during the degradation of the polymer and could adversely affect the surrounding cells.

To avoid the prospective leakage of toxic organic solvents from the electrospun coaxial nanofibers we have dissolved the polycaprolactone in a mixture of formic acid and acetic acid. As shown in the figures 4A, and 5B the electrospinning process was very stable, and in all three types of experiments it generated smooth nanofibers with a regular diameter. The electrostatic field was responsible for the observed decrease in the coaxial fiber diameter PCL\textsubscript{5}/PCL\textsubscript{10} as compared to the non-coaxial fiber PCL\textsubscript{10}. The electrostatic field was tuned the way that it would stretch the coating solution and guarantee optimum friction for stretching of the core. The observed further decrease in PCL\textsubscript{5}/PCL\textsubscript{Ag} fiber diameter as compared to
PCL₅/PCL₁₀ fibres corresponds to the presence of silver. Metallic silver is more conductive to electrical charge than popular in the industry copper. Hence, the added silver that was uniformly distributed in the solution by prior ultrasound-homogenization, increases the conductivity of the solution.

The applied in our experiments mixture of the solvents is very well suited for biological use as they have a much lower toxicity, when compared to commonly described in scientific papers organic solvents such as THF, DFM, chloroform, or methanol. Both solvents, that we used, could easily enter basic cellular metabolic pathways [24]. Lack of toxicity of the resulting tissue scaffold-replacement was confirmed by the MTT assays. The prepared by us bio-scaffold materials could potentially be used in surgery. The described new materials form a good substrate as experimental tissue scaffolds for preparation of experimental artificial organs, by using both induced-pluripotent stem cells (iPS) and also transdifferentiation techniques [25-27]. Their coaxial structure allows for embedding in the core medicinally-active substances. Animal in vivo studies are necessary to confirm the biocompatibility of the prepared nano-biomaterials.

The development of biomaterial-based artificial tissues offers protection form an accidental transfer of viral infections (marked risk in case of human- or animal-derived donor tissues). Viruses may contribute to carcinogenesis, like for example human papillomaviruses (cervical and oral cancer) [28, 29], Human T-cell Leukemia Virus (hairly cell leukemia) [30, 31] etc. Like in some other diseases [32], it is however sometimes difficult to clearly associate the given pathogen with cancer etiology. On the other hand, many viruses preferentially, or exclusively replicate in dividing cells; hence their components are potentially becoming an important source/inspiration in the search of drugs that preferentially target cancer stem cells [33-36]. Beside viral methods, some clinically tested as well as new drugs with preferential toxicity towards cancer stem cells are increasingly becoming available [37-41]. Such tasks are
facilitated by recent progress in methodologies that allows for better detection and monitoring of cancer stem cells [25, 26, 39].

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5.0 Literature


Figure legends:

Figure 1: *Diagram of the applied coaxial electrospinning process.*

Figure 2: *Conversion of tetrazolium salt to formazan.* The reaction shows the principle of MTT-assay

Figure 3: *The analysis of the obtained with our proces polycaprolactone nanofiber.* FTIR for polycaprolactone nanofiber (A), XRD spectrum for nanofiber polycaprolactone (B), and EDS for silver nanoparticles (C).

Figure 4: *Assessment of the electrospinned nanofibres.* The structure of the polycaprolactone nanofibers (no dopant added) (A). The structure of silver nanoparticles used as a dopant in some coaxial fibres. Images taken using a scanning electron microscope (SEM) (B).

Figure 5: *Examples of the nanofibres generated by electrospin, as assessed by TEM.* The structure PCL10 nanofiber obtained with a standard electrostatic field electrospin (A). The core-shell PCL5/PCL10 nanofibers obtained with coaxial electrospinning (B). Photographs taken using a transmission electron microscope (TEM).

Figure 6: *Examples of silver-nanoparticle-doped nanofibres generated by electrospin.* The structure of the core-shell PCL₅/PCL₄Ag nanofibers observed in the TEM (BF mode) (A). The structure of the core-shell PCL₅/PCL₄Ag nanofibers observed in TEM (HAADF-STEM mode) (B). Images of silver nanoparticles are clearly visible in both pictures.

Figure 7: *The structure of silver nanoparticles used to generate the doped coaxial nanofibres.* Photographs were taken using a high resolution TEM in Fourier Transform mode (FFT), inverse Fourier transform mode (IFFT), the BF mode, and HREM mode. See individual reflections silver FFT correspond to the planes (200), i.e. the FFC silver nanorstructures.

Figure 8. *PCL₅/PCL₁₀ are nontoxic, and the support well growth of human cells.* An example of cell growth on PCL5/PCL10: confocal microscopy micrograph of NHDF cells stained with propidium iodide (DNA) that were grown for 96 h on the nanofibers PCL5/PCL10 (A). The evaluation of NHDF cell survival by MTT assays (B).
Hudecki et al., Figure 5
Hudecki et al., Figure 7