3. Polymer Nanofibers Applied in Regenerative Medicine

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Outline

3.1. The concept and scope of own research of polymer nanofibers for application in regenerative medicine

The preceding parts of the work have drawn attention to the special importance of development tendencies of the medical bionic implant/artificial organs market, orthopaedic devices market, orthopaedic soft tissue repair market, orthopaedic trauma fixation devices market, orthopaedic soft tissue repair market, 3D Printing in Medical Applications Market and the biomaterials market and to the special importance, in this context, of the development of the polymer nanofibers market as a vital part of the nanomaterials market gaining increasing significance for technical protection in multiple medical activities, in particular in regenerative medicine. This entails numerous investigations concerning new groups of engineering materials, both, metallic, ceramic and polymer materials, as well as the completely new generations of composite and nanostructural materials. An example can be porous materials or hybrid constructions with porous zones enabling the growth of living tissues after implantation, biological-engineering materials, accelerating and facilitating the growth of living cells after implantation of such material, e.g. to heal extensive wounds and burns, to support the restoration of natural tissue and for use in membranes by which the development of actual tissues can be controlled, as well as polymer nanofibers and nanoporous materials consisting of nanofibers, as a special kind of a nanocomposite material, and such concept has been presented in the preceding parts of this work. Porous polymer materials are a special group here, in which a reinforcing phase are nano- and sometimes microfibers, and the matrix function is played by the air. Such materials should feature high porosity, air permeability, absorptivity, barrierity, bacteriocidity, antifungalness, biocompatibility, biodegradability, non-toxicity, a possibility of releasing medicinal agents in a controlled way, appropriate mechanical strength and the structure supporting regeneration. The application of such materials in a therapeutic practice requires numerous interdisciplinary studies, as well as development and implementation works. In the light of the above information, knowledge about polymer nanofibers and their manufacturing technologies, which are the main topic of interest of this work, is becoming more important, both theoretically and practically. An opportunity to overcome the problems and challenges arising is to create such new nanostructural materials for medical applications which, compared to conventional solutions, will not only replace the function of the lost tissue,

but will also actively influence the tissue environment, by sustaining and accelerating the naturally occurring regeneration processes.

The primary aim of this part of the work is to investigate the properties of the obtained single-component and double-component and composite nanofibers in terms of their applicability as scaffolds in tissue engineering [1]-[12]. A research thesis was proposed concerning the possibility of obtaining composite nanofibers with a bactericidal coating and a bioactive core for tissues scaffolds for application in tissue engineering. Tissue engineering inscribes itself in the area of regenerative medicine and seeks innovative methods of restoring a natural tissue and provides an alternative solution to the currently used conventional treatment methods. The nanomaterials obtained underwent the following examinations to confirm the assumed aim of the work: infrared spectroscopy FTIR, wide-angle X-ray scattering (WAXS), BET (Brunauer, Emmett and Teller), Langmuir specific surface area and DTF porosity assessed with the gas adsorption method, in transmission electron microscope (TEM), scanning electron microscope (SEM), fluorescence microscope, antibacterialness and antifungalness investigations and examinations of biological properties in vitro. The influence of conditions of obtaining nanofibers, environmental conditions and properties of a solution influencing the diameter of fibers obtained is shown schematically in Figure 3.1.

It is the aim of this work to fabricate (create) completely bioresorbable tissue scaffolds using polymer nanofibers which, owing to their properties, support natural regeneration processes of a natural tissue and temporarily substitute its function, by undergoing gradual degradation until recovering its full functionality. It is not a sufficient condition, however, to restore the structure of a natural tissue, as it is required from a material from which scaffolds are made, to be nontoxic, depending, in particular, on the solvents used for conversion into nanofibers. If toxic solvents are used, there is a risk that they are transmitted into an organism, even if only in residual content. A solvent, in the entire period of degradation, may adversely affect the surrounding cells, which may lead to changes at the molecular level of cells, therefore poses a risk of carcinogenesis. For this reason, special emphasis was laid on the necessity to use non-toxic materials only, including solvents, which, even if they enter an organism as a result of polymer degradation, will be metabolised to final compounds such as water and carbon dioxide and will not create a risk of carcinogenesis.

Many more factors fundamental for the quality and properties of polymer nanofibers need to be taken into account to create double-component nanofibers. Two solutions need to be used

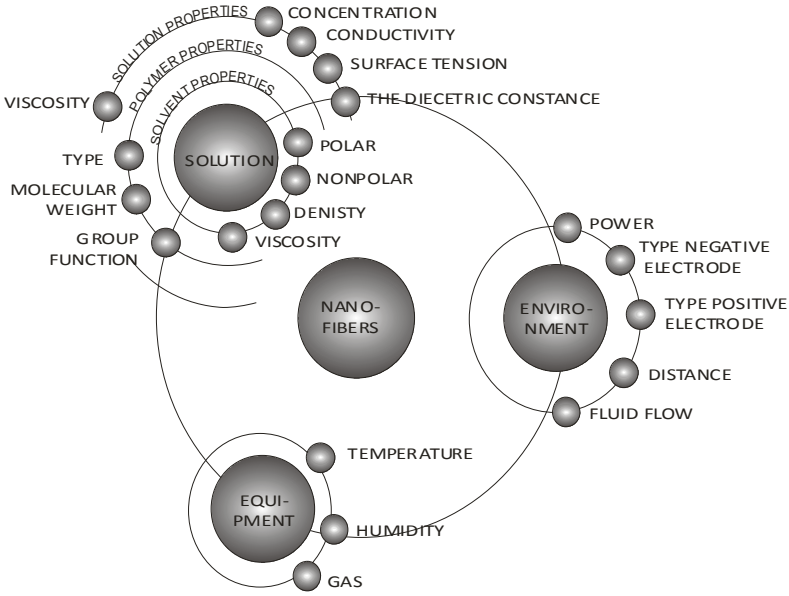

Figure 3.1. *Graphical presentation of process conditions, environmental conditions and properties of solution influencing the diameter of fibers obtained*

in co-axial electrospinning. The use of two solutions with different properties allows to isolate materials sensitive to atmospheric conditions by closing them in a core surrounded with a protective coating, by encapsulating various medicinal agents, enzymes or DNA in protective coating, and by determining the decomposition rate of the external coating, by releasing the encapsulated substances in specific time and at a specific rate, by obtaining new composite materials used, notably, as a reinforcing phase, which are a combination of a high-strength core with good external coating wettability in contact with a matrix material, by producing 3D scaffolds for the purpose of tissue engineering. Two solutions with differing properties are employed most frequently in coaxial electrospinning. For this reason, the number of conditions to be considered to achieve core-shell nanofibers, is doubled as compared to the standard electrospinning process, which substantially hinders to control the process. Moreover, interactions taking place between the core solution and the shell solution once they touch, which takes place at the end of the nozzle, and which additionally complicates the fabrication of core-coating nanofibers. Figure 3.2 shows schematically the conditions underlying the creation of core-shell nanofibers, including such acting on the core solution and external shell solution, as well as interactions between the solutions.

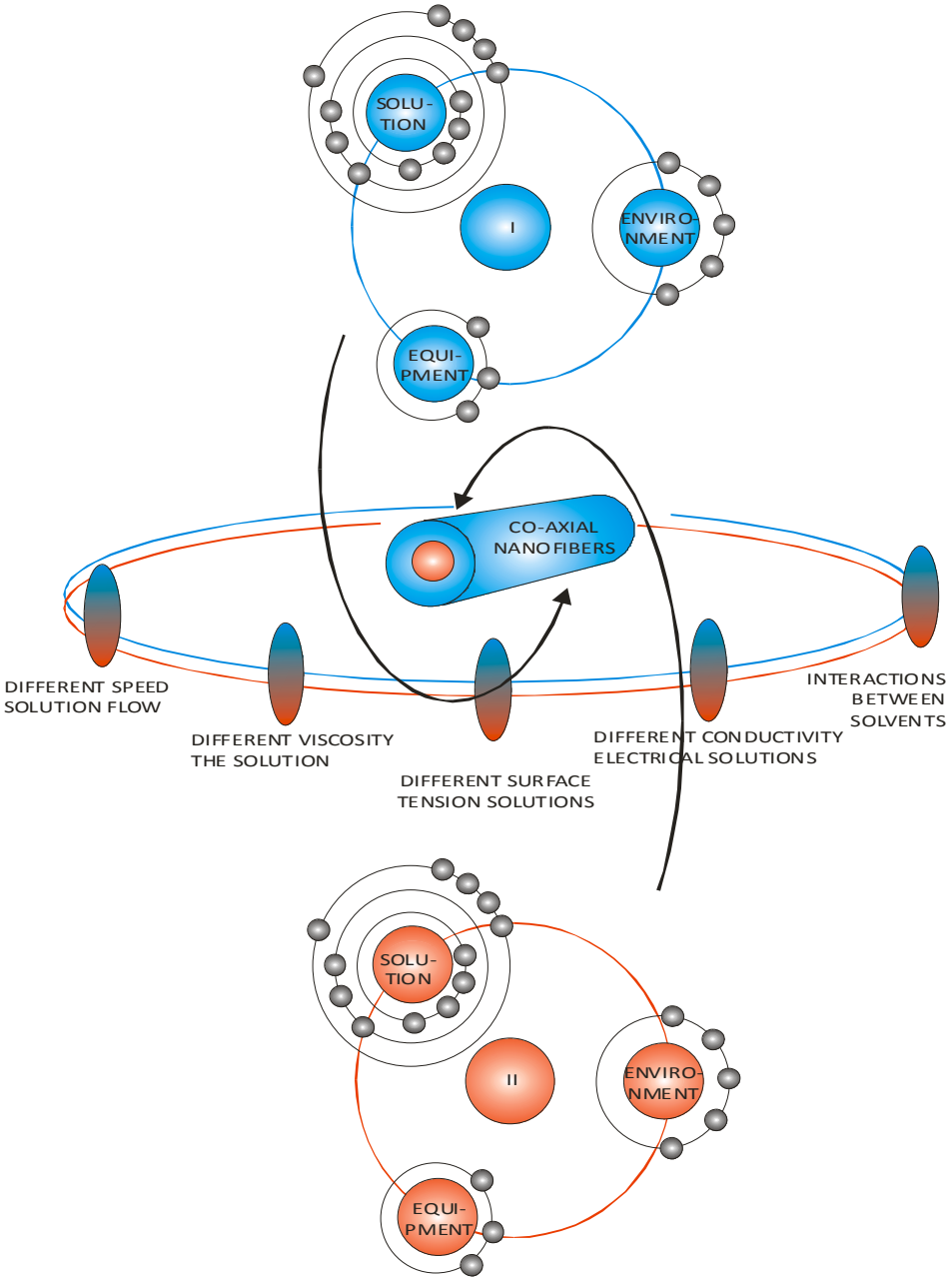

Figure 3.2. *Conditions underlying the creation of core-shell nanofibers: conditions influencing the core solution are shown in blue, conditions influencing the external shell solution are shown in orange, differences and interactions between solutions are shown in orange; own study*

3.2. Technological conditions and methodology of own research into polymer nanofibers

The following was used in order to investigate the influence of fabrication conditions and properties of solutions on the structure and properties of nanofibers: PCL (polycaprolactone) with M_w = 70,000-90,000 g/mol and M_w = 45,000 g/mol by Sigma Aldrich and the chemical reagents: 99.95% acetic acid, 99.95% formic acid, 99.95% dimethyl sulfoxide (DMSO) by Sigma Aldrich, 99.95% tetrahydrofuran (THF), methanol and chloroform by Chemland. The molecular mass of polymers was examined by means of gel permeation chromatography (GPC).

Polymer solutions with the content of 2-10%, containing polycaprolactone without additives, was prepared using polycaprolactone (PCL) with the molecular mass of M_w = 70,000-90,000 g/mol in a mixture of hydrochloric and formic acid solvents with a mass ratio of (70:30). For this purpose, the weighed polymer material was introduced into the prepared mixture of solvents and dissolved for 12 hrs using a magnetic stirrer by Chemland.

The following was prepared with polycaprolactone (PCL) with the molecular mass of M_w = 70,000-90,000 g/mol:

a) a polymer solution with the fraction of 10% in a mixture of chloroform and methanol with a mass ratio of (70:30),
b) a polymer solution with the fraction of 10% in a mixture of tetrahydrofuran and dimethyl sulfoxide with a mass ratio of (70:30),
c) a polymer solution with the fraction of 10% in a mixture of hydrochloric and formic acid with a mass ratio of (70:30).

A dependency 1 for calculating the necessary fraction of polymer materials with the accuracy of 0.01 g and of solvents measured according to their density was used for preparing the solutions. Dissolving was carried out in polypropylene or glass containers with the volume of 125 ml with a cap.

$$C_p = \frac{m_s}{m_r} \cdot 100\% \tag{3.1}$$

where:

C_p – fraction, %,

m_s – mass of the dissolved substance, g,

Table 3.1. *Potential and attractiveness criteria*

Criterion	Objective values (potential)	Weight
K_P1	conductivity of 10% of solutions: high – 10 points low – 1 point	0.20
K_P2	viscosity of 10% of solutions: high – 10 points low – 1 point	0.20
K_P3	diameter of fibers achieved: diameter of 51% of fibers below 500 nm – 10 points diameter of 51% of fibers above 500 nm – 1 point	0.15
K_P4	presence of defects: high – 10 points high content of existing defects – 1 point	0.30
K_P5	process stability (electrospinning ability): very good – 10 points insufficient – 1 point	0.15
Criteria	**Subjective values (attractiveness)**	
K_A1	toxicity of mixture of solvents: non-toxic –10 points carcinogenic – 1 point	0.25
K_A2	evaporation rate of mixture of solvents used: high – 10 points low – 1 point	0.25
K_A3	dissolution rate of polymer by the mixture of solvents used: to 1 h at room temperature – 10 points to 10 h at room temperature – 1 point	0.10
K_A4	potential range of application at industrial scale: high – 10 points low – 1 point	0.20
K_A5	availability of solvents: high – 10 points low – 1 point	0.20

m_r – mass of the solution, g.

The polymer solution, from which fibers were obtained, was selected in the investigations with the method of weighted points with the potential and attractiveness criteria used (Table 3.1-3.4).

Table 3.2. *Multicriteria analysis of attractiveness of particular PCL solutions*

Criteria	Weight	Solution obtained from mixture (70:30) of		
		hydrochloric and formic acid	tetrahydrofuran and dimethyl sulfoxide	chloroform and methanol
POTENTIAL				
K_P1	0.2	10	6	8
K_P2	0.1	9	6	7
K_P3	0.3	8	5	0
K_P4	0.2	8	6	2
K_P5	0.2	5	4	4
ATTRACTIVENESS				
K_A1	0.25	8	2	2
K_A2	0.1	7	2	2
K_A3	0.15	5	8	10
K_A4	0.25	10	2	2
K_A5	0.2	8	7	10

A PCL solution obtained from a mixture of formic acid and hydrochloric acid at a rate of 70:30 m/m (A), ranked in the most promising quarter of the matrix (wide-stretching oak), has the highest level of attractiveness and potential (Fig. 3.3). The other solutions (B) and (C) were rejected from further investigations due to the properties obtained.

The Electro–Hydrodynamic Atomization 2.2D – 500 device by Yflow Nanotechnology Solutions equipped with a working chamber, control panel, infusion pumps with flow adjustment for µl/min and ml/h, two systems maintaining solution temperature and nozzles for standard and coaxial electrospinning was used in order to transform the solutions obtained into fibers. Nanofibers were deposited during electrospinning onto a flat collector dimensioned

Table 3.3. *Analysis results of particular PCL solutions*

Criteria group	Criteria	Weight	Solution obtained from mixture (70:30) of		
			hydrochloric and formic acid	tetrahydrofuran and dimethyl sulfoxide	mixture of chloroform and methanol
POTENTIAL	K_P1	0.20	2.0	1.2	1,6
	K_P2	0.20	0.9	0.6	0,7
	K_P3	0.15	2.4	1.5	0
	K_P4	0.30	1.6	1.2	0.4
	K_P5	0.15	1.0	0.8	0.8
	SUM		**7.9**	**5.3**	**3.5**
ATTRACTI-VENESS	K_A1	0.25	2	0.5	0.5
	K_A2	0.25	0.7	0.1	0.1
	K_A3	0.10	0.75	1.2	1.5
	K_A4	0.20	2.5	0.5	0.5
	K_A5	0.20	1.6	1.4	2
	TOTAL		**7.55**	**3.7**	**4.6**

Table 3.4 . *Statement of analysis results of particular PCL solutions*

Size	Solution obtained from mixture (70:30) of		
	hydrochloric acid and formic acid	tetrahydrofuran and dimethyl sulfoxide	chloroform and methanol
SYMBOL	A	B	C
POTENTIAL	7.9	5.3	3.5
ATTRACTIVENESS	7.55	3.7	4.6

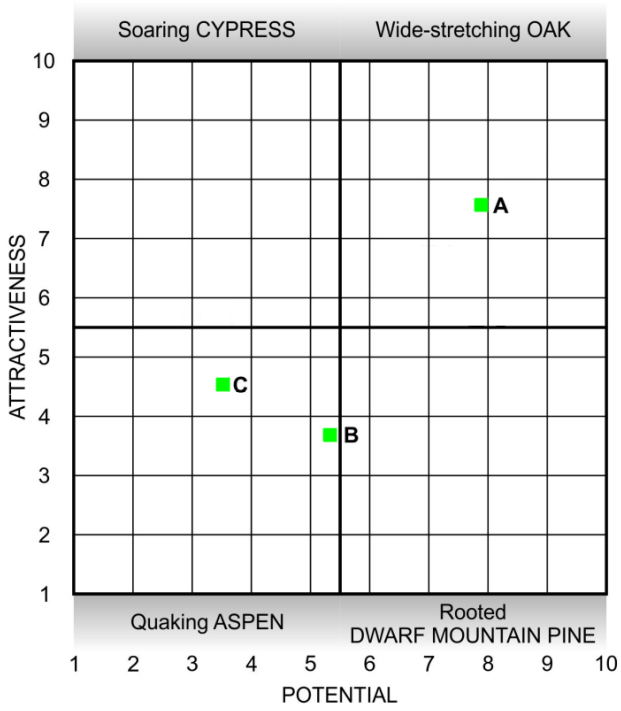

Figure 3.3. *Graphical representation of selected PCL polymers solutions' potential and attractiveness*

40x40 cm or onto a rotating collector with the width of 40 cm and diameter of 20 cm each time for 0.25-12 h. The solutions obtained were subjected to the activity of an electrostatic field in the conditions described in Table 3.5, by converting the solutions obtained into differently structured fibers.

The properties and application possibilities of polymer nanofibers can be improved prior to their surface treatment by introducing additives into polymer solutions providing bioactive microbiological properties, also into a solution with the fraction of 10% of polycaprolactone (PCL) with the molecular mass of M_w = 70,000-90,000 g/mol in a mixture of hydrochloric acid and formic acid with a mass ratio of (70:30); macromolecular chitosan with M_w = 100,000-300,000 g/mol, silver nitrate $AgNO_3$ or AlphaSan was, respectively, introduced with the fraction of 1-5%. In order to prepare such solutions, the weighed polymer materials and additives were introduced into the prepared mixture of solvents and dissolved for 12 hrs in polypropylene or glass containers with the volume of 125 ml with a cap. The polymer solutions

Table 3.5. *Conditions of obtaining single-component PCL, double-component and composite nanofibers of the core-coating type*

Fiber type		Single-component	Double-component	Core-coating
Type of nozzle applied		standard nozzle		co-axial jet
Process type		single-stream process		co-axial process
Solution flow rate, ml/h	of core	1.00		0.04-0.50
	of coating	not applicable		0.3-1.0
Type of collector applied		flat 40x40 cm rotating with diameter of 20 cm and length of 40 cm		flat 40x40 cm
Collector rotational speed, rev./min		200, 500, 600, 1400	500	not applicable
Electrostatic voltage, kV/cm		0.95-1.00	0.95-1.63	0.95-1.20
Solution temperature, ^{o}C		25		
Gas temperature in working chamber, ^{o}C		23		
Gas humidity in working chamber, %		20-30		

containing silver nitrate $AgNO_3$ or AlphaSan, prior to adding polycaprolactone, underwent sonification using a Labindex homogeniser for 5 minutes, and were left for 12 hours after adding PCL to dissolve.

After dissolving, the solutions were placed in containers of the Yflow Nanotechnology Solutions device, where a flow rate and solution temperature was controlled and then it was subjected to the activity of an electrostatic field converting the solutions into differently structured fibers.

Core-shell composite nanofibers were also fabricated by a technique of co-axial electrospinning. The shell was prepared with a polycaprolactone solution with the molecular mass of M_w = 70,000-90,000 g/mol with a 3% additive of silver nitrate and with a solvent of hydrochloric acid and formic acid, whereas the core was made of a polycaprolactone solution with addition of 5% of low molecular hyaluronic acid in the conditions described in Table 3.5. Co-axial jets were used to obtain core-shell nanofibers in an electrostatic field enabling the

flow of two 'jet in jet' polymer solutions, in which an inner jet can be distinguished, responsible for the flow of a solution forming the core of nanofibers consisting of two parts and a surrounding external jet responsible for creating a shell surrounding the core from each side.

Solution viscosity tests were performed with a rotational viscometer, tests of electrical conductivity of solutions with a conductometer, structure tests with Scanning Electron Microscopy (SEM) and Electron Transmission Microscopy (TEM). Investigations with the adsorption method allow to characterise porosity and BET specific surface area. The total volume of the substance absorbed on the adsorbent equals the sum of the substance in all layers and is described with the Brunauer, Emmett and Teller equation (3.2), called the BET isotherm equation:

$$\frac{p}{V(p_0 - p)} = \frac{1}{V_n c} + \frac{(c-1)p}{V_n c\, p_0} \tag{3.2}$$

where:

p_0 – saturated vapour pressure of adsorbate,

V – volume of gas adsorbed under pressure p,

V_n – volume of gas covering, in a monomolecular way, the surface layer,

c – a constant exponentially linked to the difference between adsorption heat in the first layer q_o and adsorption heat n further layers q, which, for simplification, was adopted as constant and equal to condensation heat q_k. Considering that $D\,q_o > D\,q_k$, the adsorption isotherm in all the cases assumes the shape of the 2nd isotherm type and signifies that physical adsorption is created, i.e. creation of a multi-molecular layer of nitrogen.

3.3. The results of own investigations of polymer nanofibers

The outcomes of own investigations of single- and double-component polymer nanofibers as well as of long-resorbable oval composite nanofibers with a bioactive core and a bactericidal coating are presented in this part of the work.

When the fraction of caprolactone is increased in a solution with three mixtures of solvents, this has increased solution viscosity (Fig. 3.4). The highest increase in viscosity is characteristic for a solution in the mixture of tetrahydrofuran and dimethyl sulfoxide from 7 to 933 mPa·s, and a much smaller increase occurs in the case of a mixture of hydrochloric acid and formic acid from 9 to 133 mPa·s.

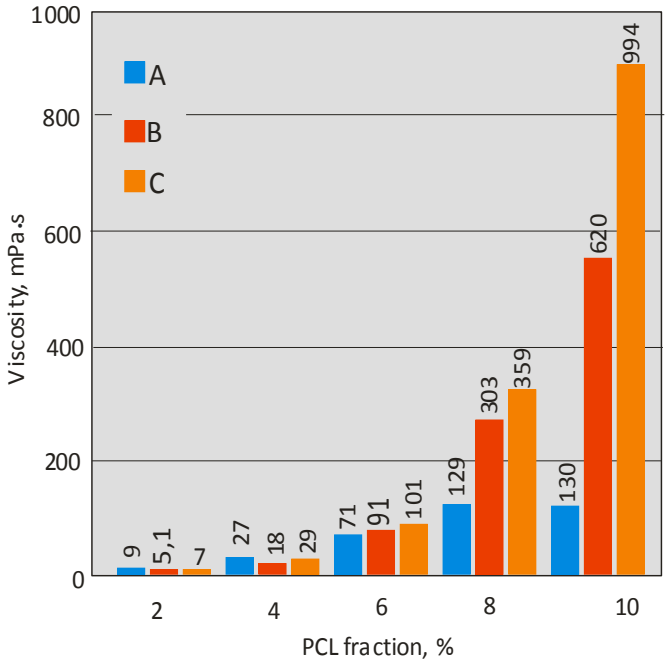

Figure 3.4. *Change of viscosity of polycaprolactone solution with fraction of 2-10% dissolved in mixture of hydrochloric acid and formic acid (A), chloroform and methanol (B) and a mixture of tetrahydrofuran and dimethyl sulfoxide (C)*

The viscosity of the solutions obtained is also changed by introducing the additives of chitosan, AlphaSan and silver nitrate into the initial polycaprolactone solution. If the fraction

of the abovementioned additives is increased, viscosity is increased from 133 mPa·s for a solution not containing additives to, respectively, up to 568 mPa·s, 844 mPa·s and to 2323 mPa·s for solutions containing 5% of silver nitrate, 5% of AlphaSan, and 5% chitosan (Fig. 3.5).

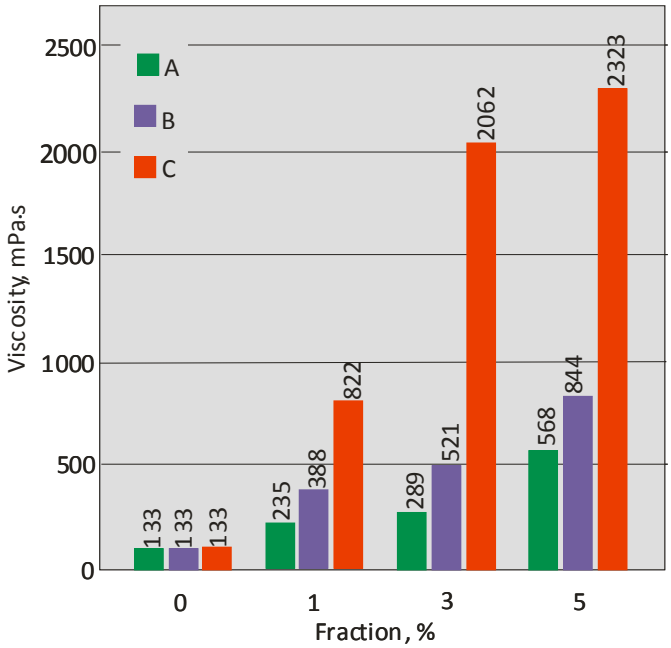

Figure 3.5. *Change of dynamic viscosity of polycaprolactone solutions (M_w = 70,000-90,000 g/mol[1]) with the fraction of 9.5-10%, obtained by dissolving PCL granulate in the mixture of formic acid and hydrochloric acid (mass ratio of 70:30) and by adding 0-5% of $AgNO_3$ (A), AlphaSan (B), chitosan (C)*

The electric conductivity of the solutions is also changed (Fig. 3.6), which decreases as the fraction of polycaprolactone, being an isolator, is rising. The initial conductivity is decreased by 66.6% from 4.5 to 1.6 μS/cm in case of PCL solution in a mixture of chloroform and methanol with the fraction of, respectively, 2 and 10% of PCL. The smallest reduction is seen

[1] The molecular mass of polycaprolactone according to manufacturers' guidelines is given in the text of, respectively, M_w = 45,000 g/mol and M_w = 70,000-90,000 g/mol. Own investigations of the molecular mass of polymers used by means of gel permeation chromatography (GPC) show differences in the molecular mass of the tested polymer materials given by the manufacturer and are, respectively, M_w = 38,107 g/mol for the first polymer and M_w = 100,720 g/mol for the second polymer.

for a mixture of hydrochloric acid and formic acid, the conductivity of solutions falls by 30.2% of the initial value of 43 to 30 µS/cm, respectively, with the fraction of PCL of 10 and 2%. The influence of silver nitrate additives, AlphaSan and chitosan on electrical conductivity of polycaprolactone solutions is diverse.

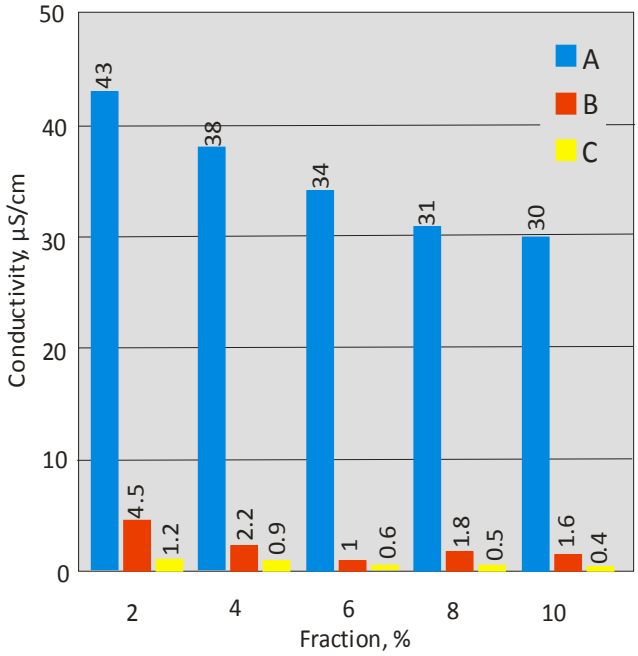

Figure 3.6. *Change of electric conductivity of polycaprolactone solution with fraction of 2-10% dissolved in mixture of hydrochloric acid and formic acid (A), chloroform and methanol (B) and a mixture of tetrahydrofuran and dimethyl sulfoxide (C)*

The electric conductivity of the solutions is increased after adding chitosan from 28 µS/cm in case of polycaprolactone not containing chitosan, to 366 µS/cm with the fraction of 5%, and silver nitrate to 840 µS/cm with the fraction of 5%, whereas it is decreased – for solutions containing AlphaSan, to 23 µS/cm for fraction of 5% (Fig. 3.7), which is the lowest electric conductivity for all the studied solutions.

The solvents used, with a varied evaporation rate, influence the obtainment of differently structured fibers. Solvents with high volatility support the creation of fibers with large diameter and disadvantageously do not support the creation of nanofibers. The slowly evaporating solvents influence the creation of nanofibers. The diameter and geometrical properties of

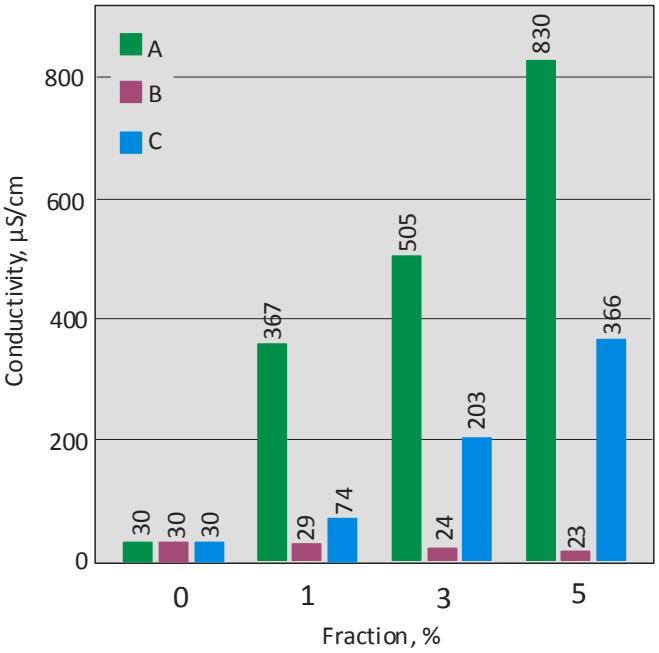

Figure 3.7. *Change of electric conductivity of polycaprolactone solutions (M_w = 70,000-90,000 g/mol) with the fraction of 9.5-10%, obtained by dissolving PCL granulate in the mixture of formic acid and hydrochloric acid (mass ratio of 70:30) and by adding 0-5% of $AgNO_3$ (A), AlphaSan (B), chitosan (C)*

polymer fibers obtained in an electrostatic field, deposited onto the surface of a flat collector, are therefore largely dependent on the mixtures of solvents employed: tetrahydrofuran and dimethyl sulfoxide; chloroform and methanol; and hydrochloric acid and formic acid (Fig. 3.8). 76% of the nanofibers obtained using a mixture of chloroform and methanol exhibit the diameter of 1.0-2.1 µm, while the remaining 24% are nanofibers, and the thinnest ones are nanofibers with the diameter of 500-600 nm, as indicated by the results of SEM investigations. Fibers with a high irregularity of diameters are formed by employing this mixture of solvents and numerous surface defects occur, the fibers are glued; the similar geometric features are exhibited by fibers obtained from a mixture of solvents of tetrahydrofuran and dimethyl sulfoxide (Fig. 3.8).

68% are microfibers with the diameter of 1.0-2.5 µm, whilst the remaining 32% are nanofibers, and the thinnest ones are nanofibers with the diameter of 400-500 nm. The fibers obtained using tetrahydrofuran and dimethyl sulfoxide are also characterised by gluing

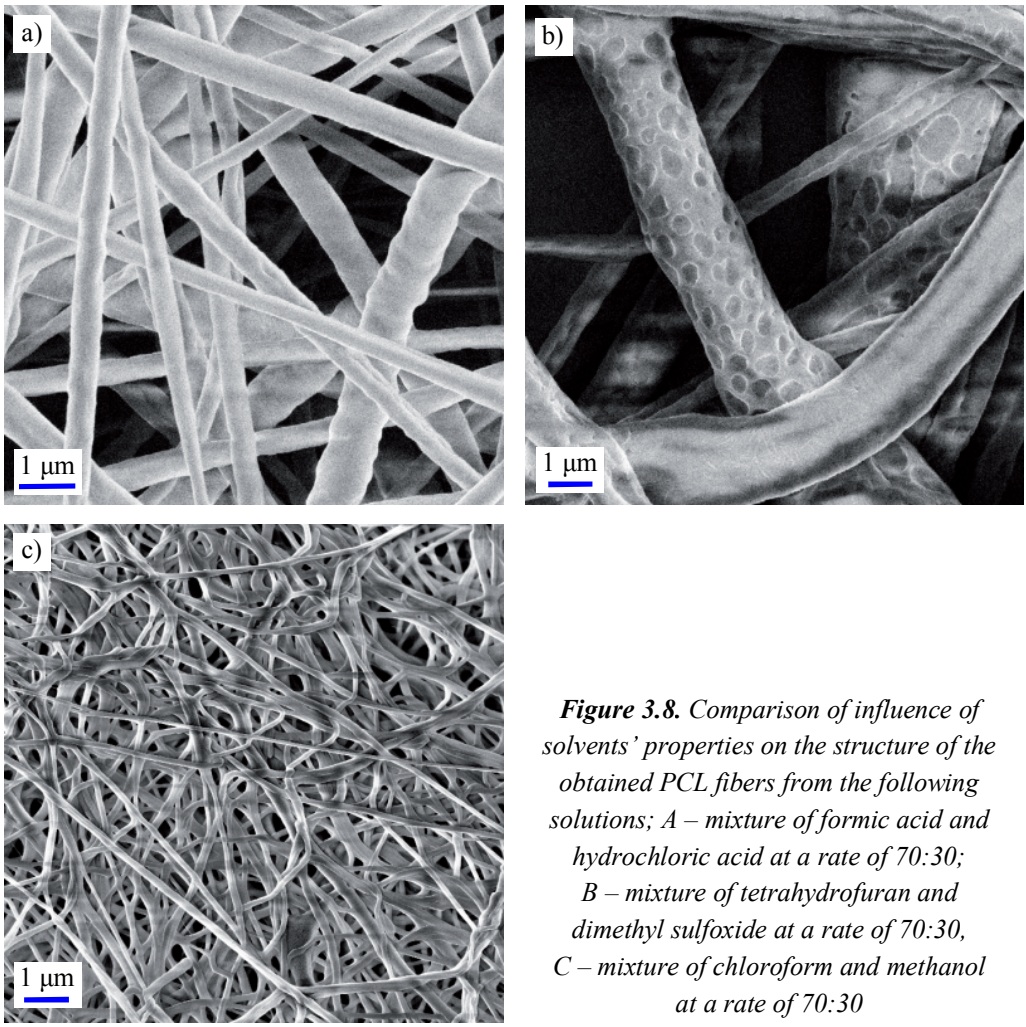

Figure 3.8. *Comparison of influence of solvents' properties on the structure of the obtained PCL fibers from the following solutions; A – mixture of formic acid and hydrochloric acid at a rate of 70:30; B – mixture of tetrahydrofuran and dimethyl sulfoxide at a rate of 70:30, C – mixture of chloroform and methanol at a rate of 70:30*

tendency and are distinctive for their high geometrical irregularity of the diameter and surface. Completely different geometrical characteristics are exhibited by fibers achieved from a polycaprolactone solution using a mixture of formic acid and hydrochloric acid (Fig. 3.8), as nanofibers with the diameter of 1-500 nm occur then only, and the thinnest ones are nanofibers with the diameter of 1-100 nm, and, among them, a fraction of ultrathin nanofibers with the diameter of 20-30 nm, not existing in other cases. The nanofibers obtained are characterised by a smaller gluing tendency as compared to other samples, and – due to the properties of the solvents used – show much smaller toxicity.

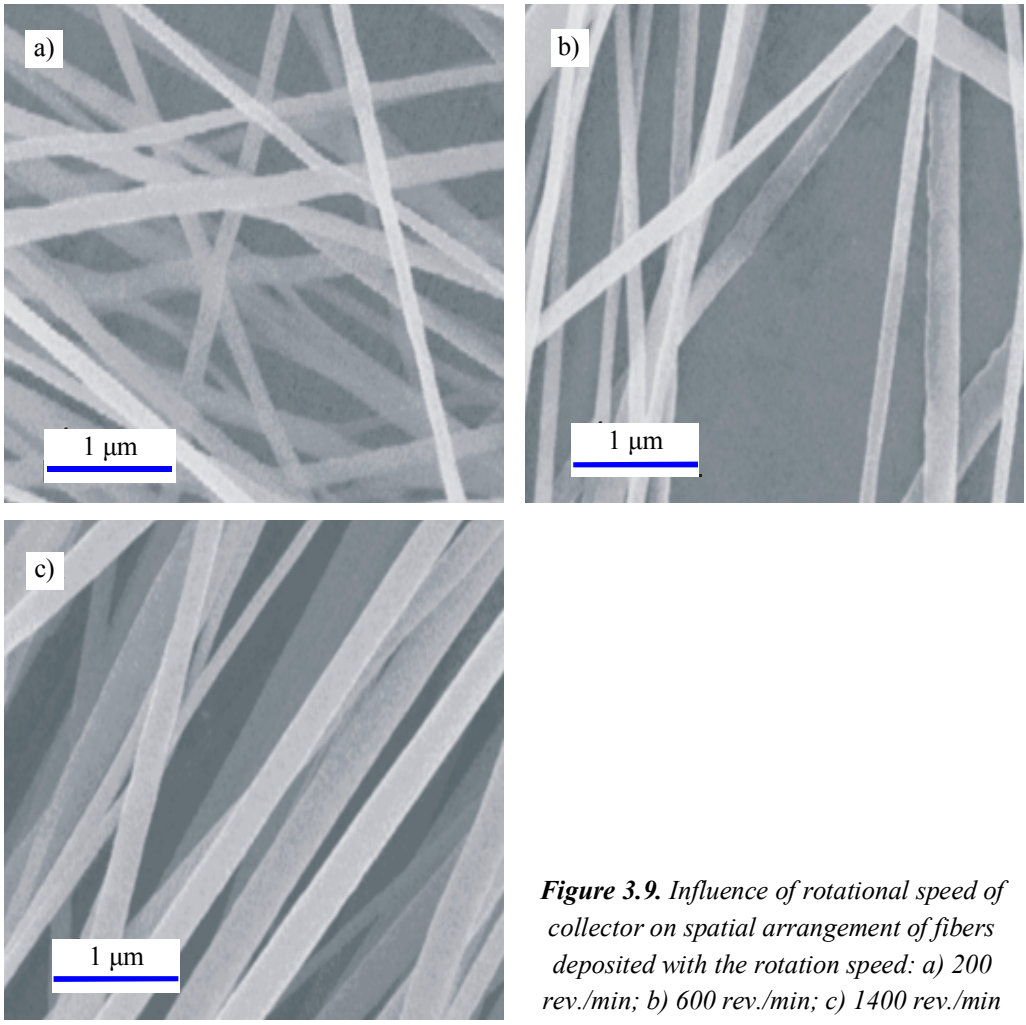

Figure 3.9. *Influence of rotational speed of collector on spatial arrangement of fibers deposited with the rotation speed: a) 200 rev./min; b) 600 rev./min; c) 1400 rev./min*

The type of the collector used (rotating or flat) and rotational speed (for rotating collectors) is highly influencing the geometrical characteristics and isotropy of nanofibers obtained from a 10% polycaprolactone solution in a mixture of hydrochloric acid and formic acid. Fibers deposited onto a flat collector are characterised by isotropy, whereas the fibers deposited onto a rotating collector are anisotropic. The deposition of fibers onto the surface of a rotating collector is highly influencing their spatial arrangement (Fig. 3.9). The diameter of the obtained fibers depends on the rotation speed of the collector. In case of fibers deposited onto a rotating collector with the rotation speed of 200 rev./min, 72% of all nanofibers are those with the

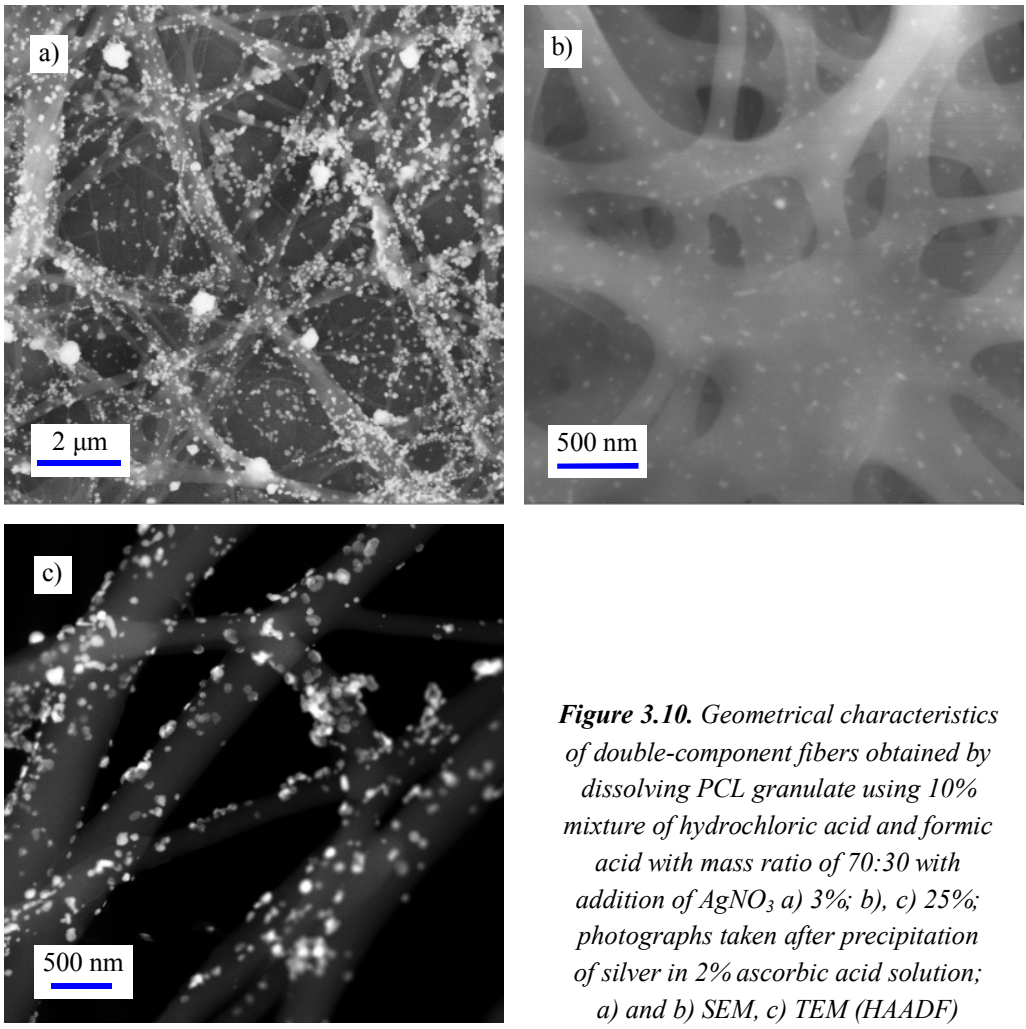

Figure 3.10. *Geometrical characteristics of double-component fibers obtained by dissolving PCL granulate using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with addition of $AgNO_3$ a) 3%; b), c) 25%; photographs taken after precipitation of silver in 2% ascorbic acid solution; a) and b) SEM, c) TEM (HAADF)*

diameter of 100-200 nm, and 68% for rotational speed of 600 rev./min. The biggest differences in the diameter of the fibers obtained with the diameter of 1 to 1000 nm occur for rotational speed of 1400 rev./min, although nanofibers with the diameter of 100-200 nm account for 50%. Fibers' anisotropy is increased as the rotational speed of the collector is increased. Opposite to the fibers obtained with a flat collector, there is no gluing tendency of fibers in case of employing a rotation collector.

The molecular mass of polymers is also considerably influencing the diameter of micro- and nanofibers obtained from a 10% polycaprolactone solution in a mixture of formic acid

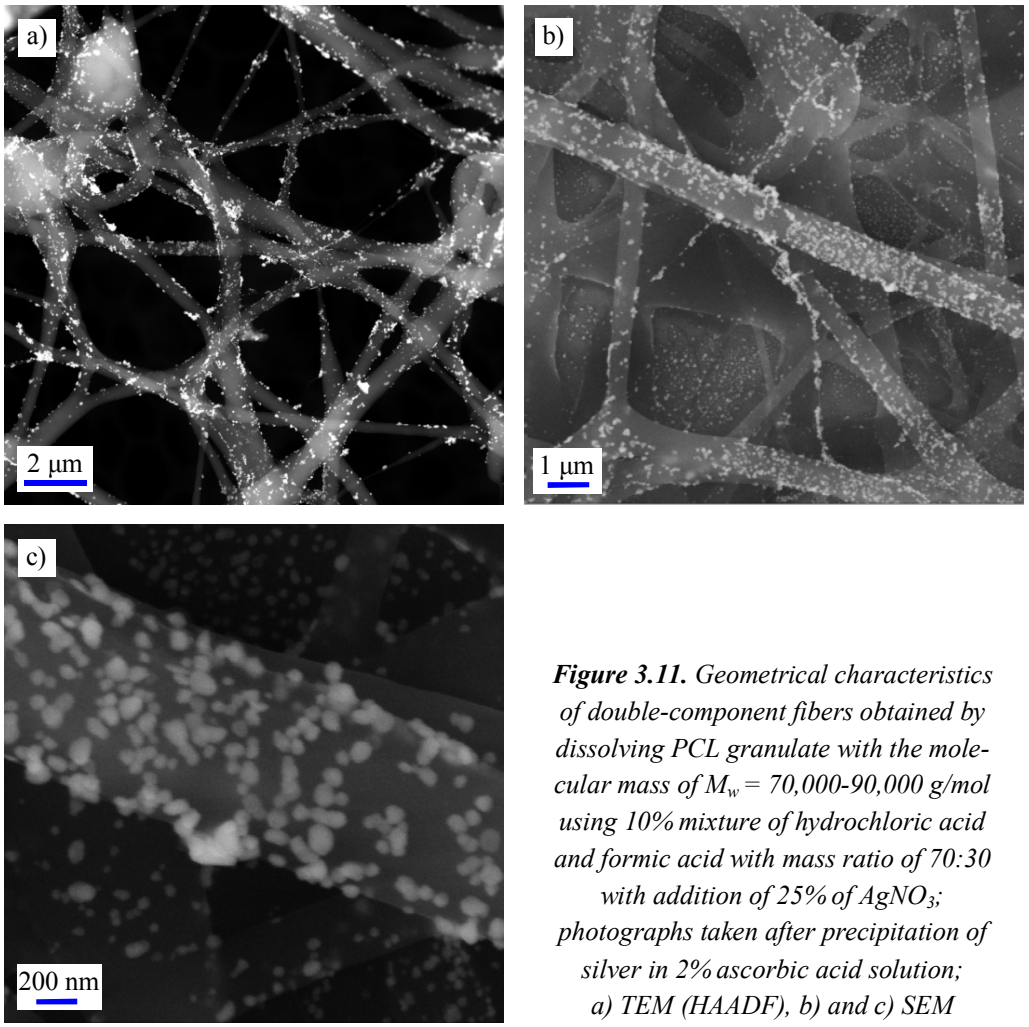

Figure 3.11. *Geometrical characteristics of double-component fibers obtained by dissolving PCL granulate with the molecular mass of M_w = 70,000-90,000 g/mol using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with addition of 25% of $AgNO_3$; photographs taken after precipitation of silver in 2% ascorbic acid solution; a) TEM (HAADF), b) and c) SEM*

and hydrochloric acid. In case of using a PCL polymer material with smaller molecular mass M_w = 45,000 g/mol, larger differences exist in the diameter of the fibers obtained in the range of 1 to 800 nm as compared to the use of a PCL polymer material with higher molecular mass of M_w = 70,000-90,000 g/mol, when fibers with the diameter of 1 to 500 nm are formed.

By introducing the additives of silver nitrate, AlphaSan and chitosan into the solutions, the diameter of the fibers produced therein is influenced (Fig. 3.10 to Fig. 3.14). If an additive of 1-5% of chitosan is introduced, the dominant diameter of nanofibers is 200-300 nm. The

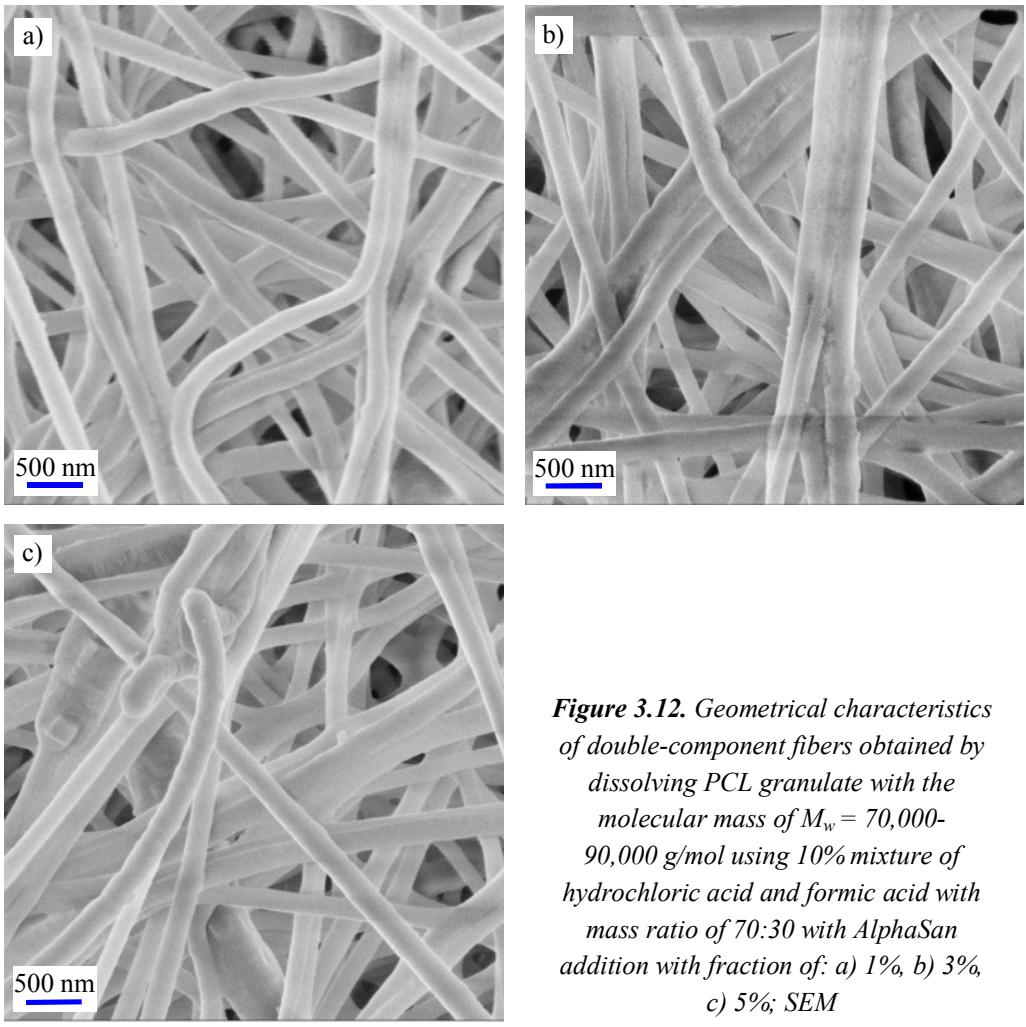

Figure 3.12. *Geometrical characteristics of double-component fibers obtained by dissolving PCL granulate with the molecular mass of M_w = 70,000-90,000 g/mol using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with AlphaSan addition with fraction of: a) 1%, b) 3%, c) 5%; SEM*

diameter of fibers is increased by increasing the fraction of chitosan, but it also causes the existence of ultrathin nanofibers with the diameter smaller than 50 nm (Fig. 3.13). Silver particles with the diameter of 20 nm exist on the surface of fibers in case of double-component fibers containing an additive of silver nitrate (Fig. 3.10 and Fig. 3.11).

The tests of porosity and of the BET, Langmuir specific surface area and of porosity with the DTF method with the nitrogen adsorption method indicate that the largest specific surface area of 8.6 m^2/g is seen for PCL fibers fabricated with a mixture of hydrochloric acid and formic acid (Fig. 3.15 to Fig. 3.23). The specific surface area of fibers obtained using a mixture

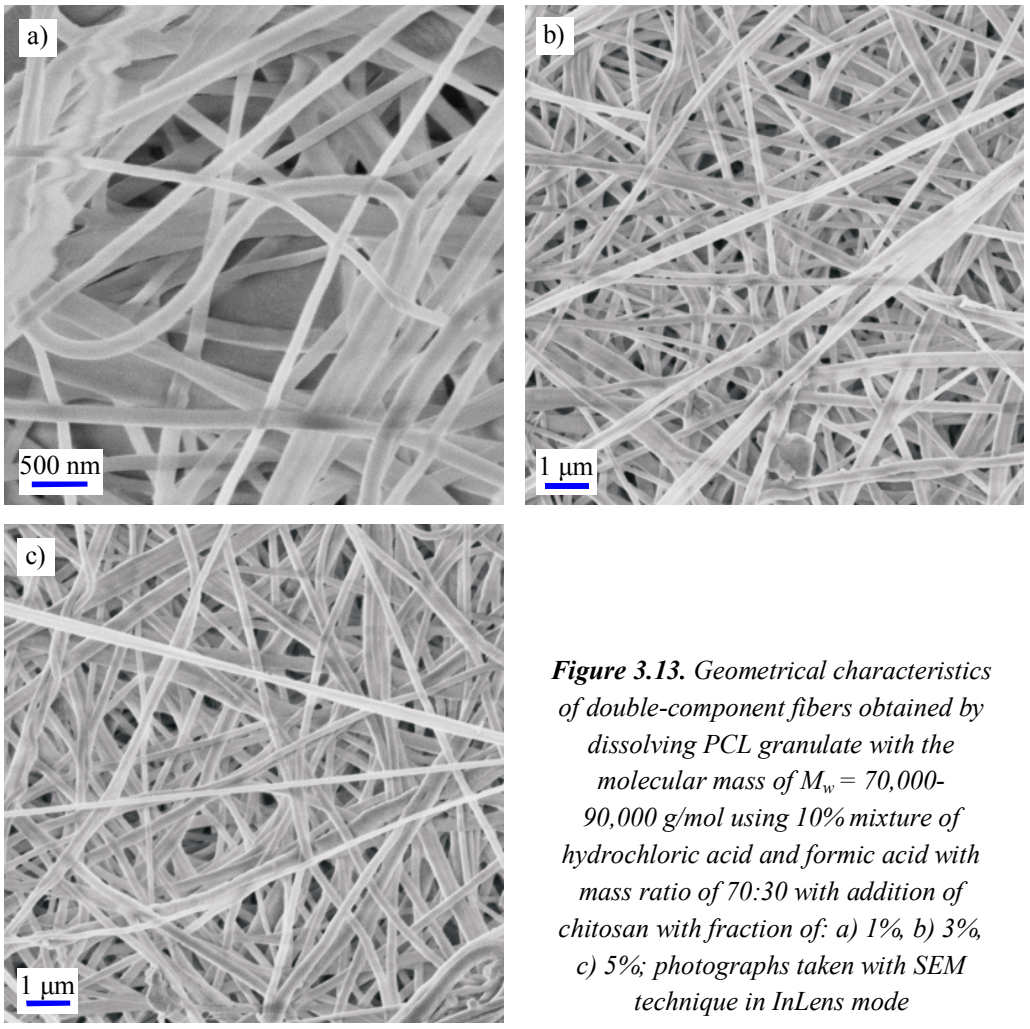

Figure 3.13. *Geometrical characteristics of double-component fibers obtained by dissolving PCL granulate with the molecular mass of M_w = 70,000-90,000 g/mol using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with addition of chitosan with fraction of: a) 1%, b) 3%, c) 5%; photographs taken with SEM technique in InLens mode*

of tetrahydrofuran and dimethyl sulfoxide is 3.1 m^2/g. The smallest specific surface area of 0.96 m^2/g is seen for the fibers obtained with a mixture of chloroform and methanol, and the width of pores is within the range of 1.5-3.4 nm. In case of the fibers obtained using a mixture of formic acid and hydrochloric, their specific surface area of approx. 0.8 m^2/g is largest for pores with the diameter of 1.6 nm.

In case of fibers obtained using a mixture of tetrahydrofuran and dimethyl sulfoxide, the diameter of pores of 1.6 nm corresponds to the specific surface area of 0.45 m^2/g. The smallest specific surface area is exhibited by fibers obtained with a mixture of chloroform and

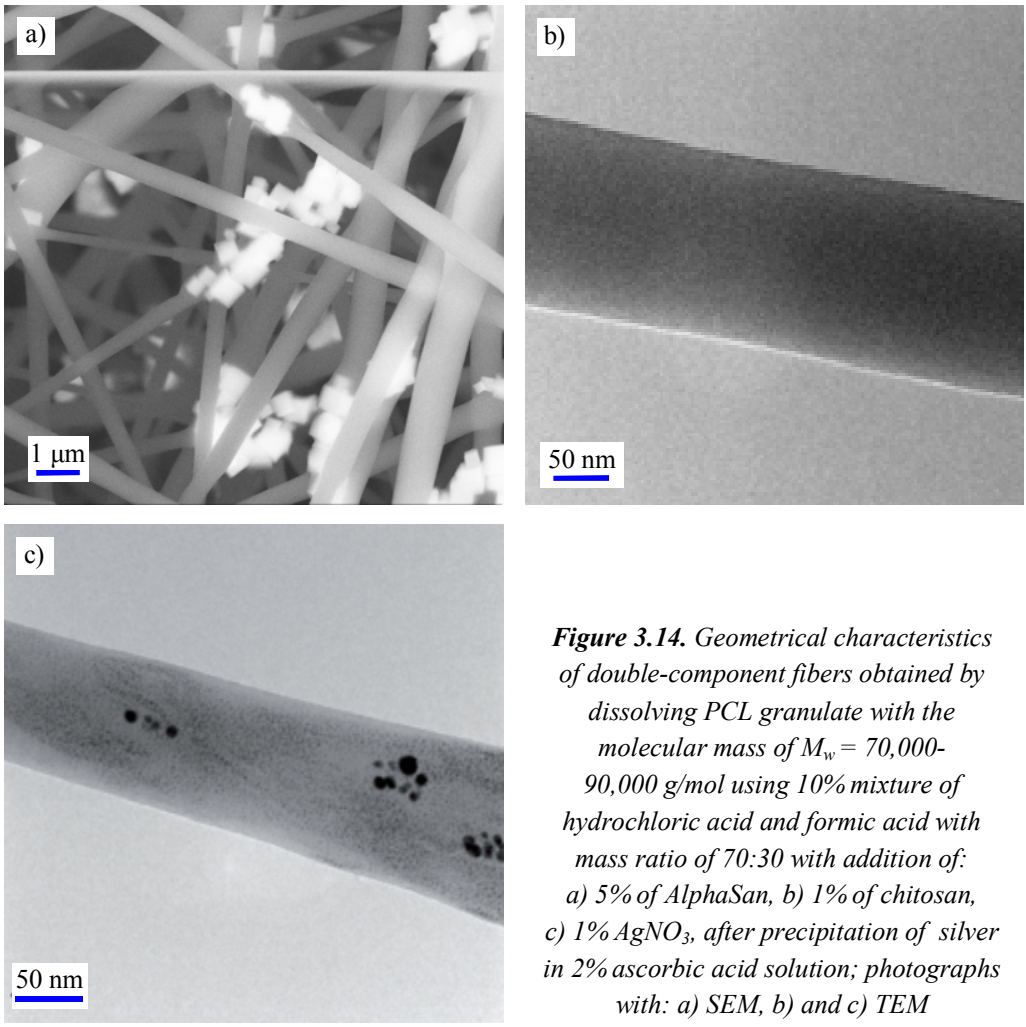

Figure 3.14. *Geometrical characteristics of double-component fibers obtained by dissolving PCL granulate with the molecular mass of M_w = 70,000-90,000 g/mol using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with addition of: a) 5% of AlphaSan, b) 1% of chitosan, c) 1% $AgNO_3$, after precipitation of silver in 2% ascorbic acid solution; photographs with: a) SEM, b) and c) TEM*

methanol, and – for the pores' diameter of 1.6 nm, it is 0.05 m^2/g. The mentioned specific surface areas correspond to the adsorption ability of nitrogen.

A specific surface area of the fibers produced is dependent on the presence of additives of silver nitrate, AlphaSan and chitosan (Fig. 3.15 to Fig. 3.23). As the fraction of silver nitrate and AlphaSan is growing, so is growing the BET and Langmuir specific surface area. A different tendency occurs if the fraction of chitosan is increased, as the specific surface area is then decreased. The highest porosity determined with the DFT method in the range of 1.4-5.4 nm for the fibers with an additive of chitosan, AlphaSan or silver nitrate is observed for fibers

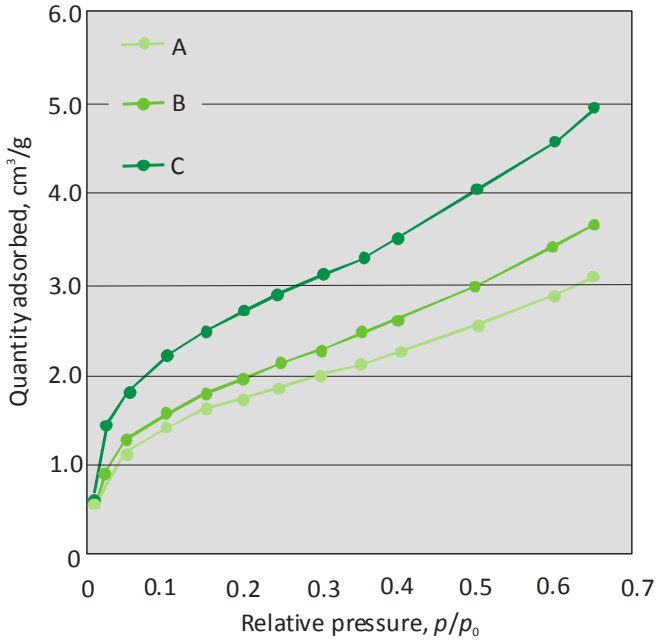

Figure 3.15. *Adsorption isotherms for PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of $AgNO_3$ with fraction of: A) 1%, B) 3%, C) 5%, after precipitation of silver with 2% ascorbic acid solution*

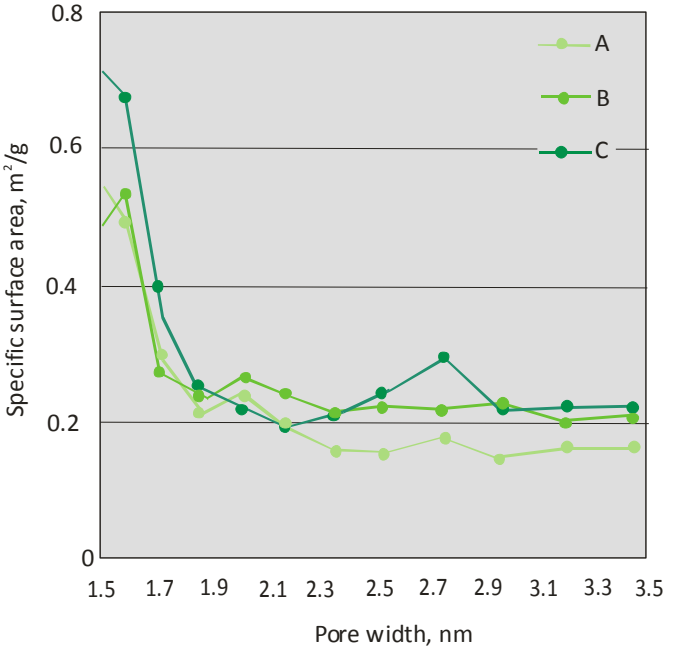

Figure 3.16. *Specific surface area of pores determined with the FTF method in an incremental way for PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of $AgNO_3$ with fraction of: A) 1%, B) 3%, C) 5%, after precipitation of silver with 2% ascorbic acid solution*

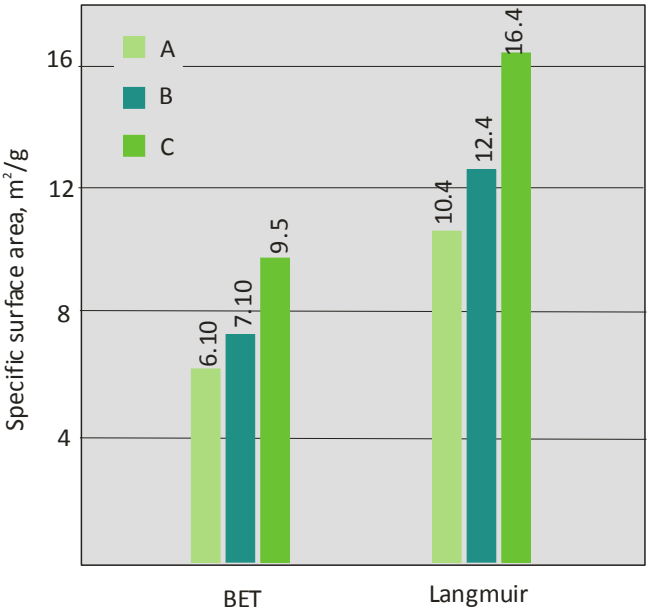

Figure 3.17. *BET and Langmuir specific surface area of PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of $AgNO_3$ with fraction of: A) 1%, B) 3%, C) 5%, after precipitation of silver with 2% ascorbic acid solution*

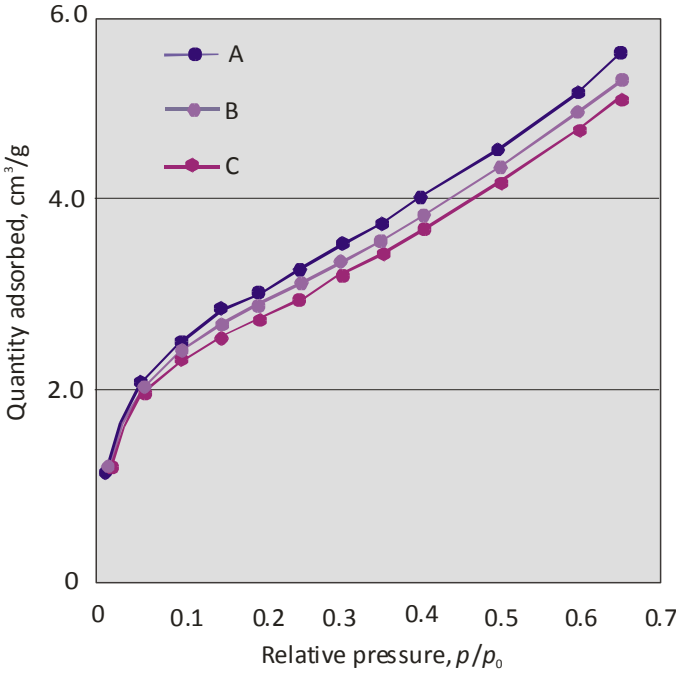

Figure 3.18. *Adsorption isotherms for PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of AlphaSan with fraction of: A) 1%, B) 3%, C) 5%*

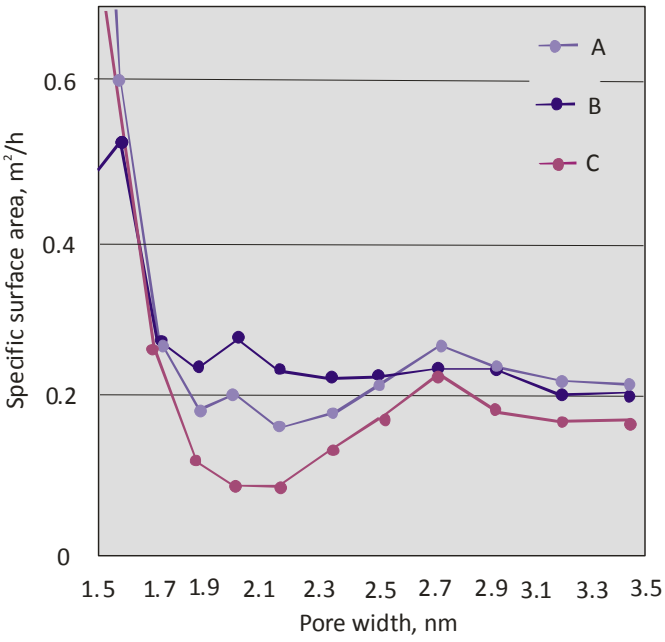

Figure 3.19. *Specific surface area of pores determined with the DTF method in an incremental way for PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of AlphaSan with fraction of: A) 1%, B) 3%, C) 5%*

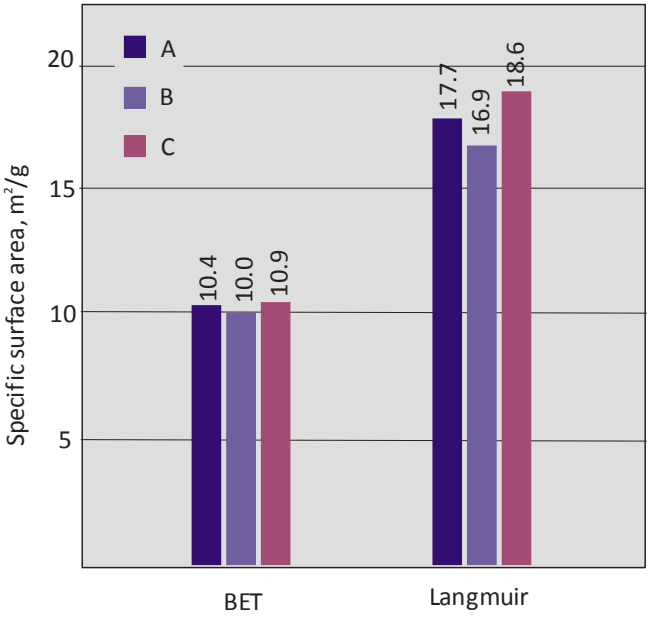

Figure 3.20. *BET and Langmuir specific surface area of PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of AlphaSan with fraction of: A) 1%, B) 3%, C) 5%*

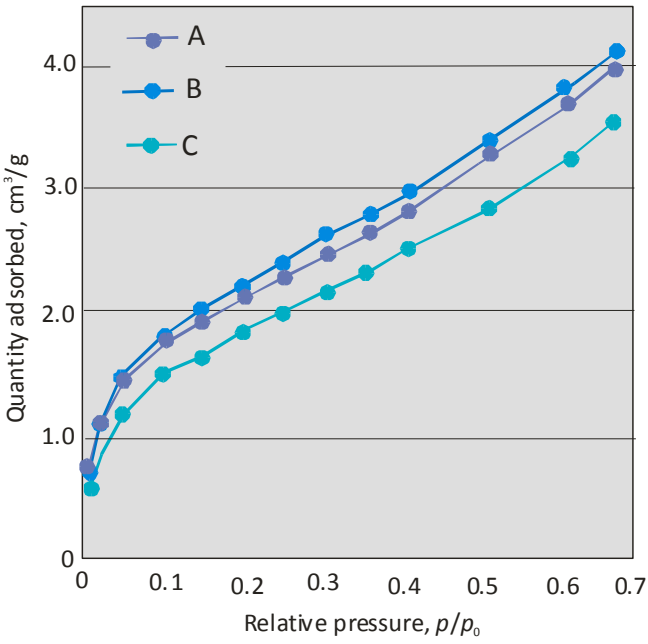

Figure 3.21. *Adsorption isotherms for PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of chitosan with fraction of: A) 1%, B) 3%, C) 5%*

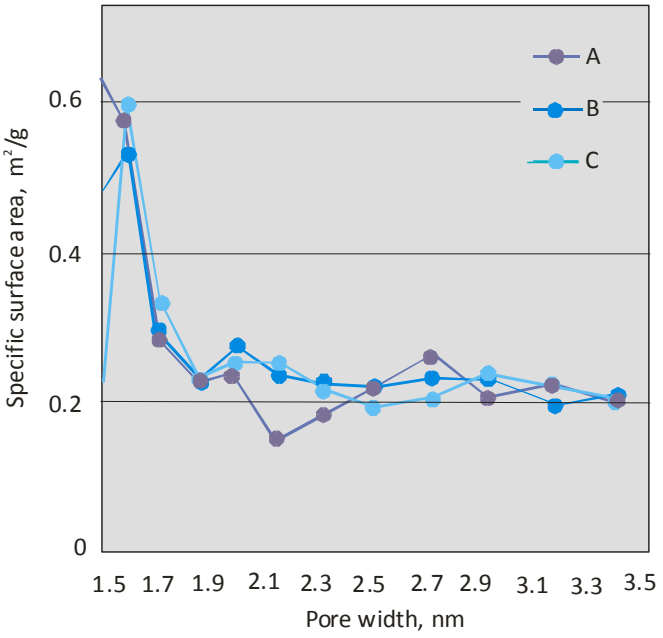

Figure 3.22. *Specific surface area of pores determined with the DTF method in an incremental way for PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of chitosan with fraction of: A) 1%, B) 3%, C) 5%*

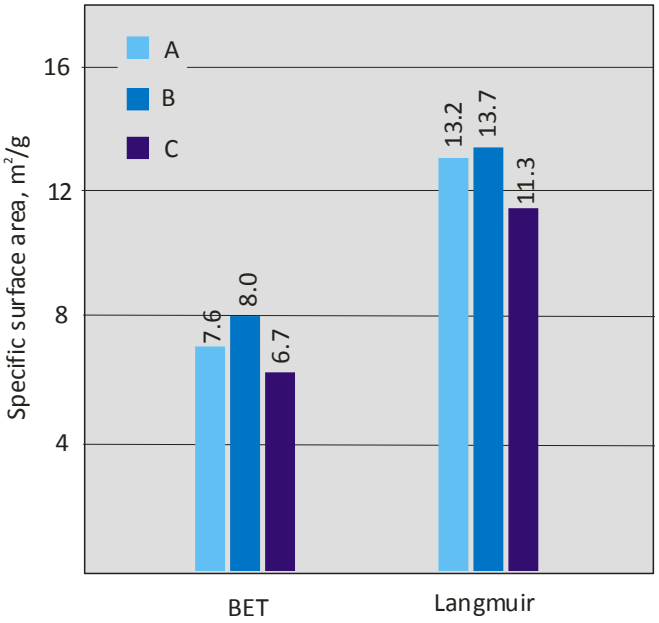

Figure 3.23. *BET and Langmuir specific surface area of PCL fibers with the molecular mass of M_w = 70,000-90,000 g/mol obtained from 10% mixture of formic acid and hydrochloric acid with mass ratio of 70:30 with addition of chitosan with fraction of: A) 1%, B) 3%, C) 5%*

containing 5% of AplhaSan. After introducing a 5% additive of AlphaSan, an area of pores with the diameter of more than 1.4 nm is 5.97 m^2/g, and pores are dominant with the diameter of 1.58 nm, with their corresponding specific surface area of 0.66 m^2/g.

For a 5% additive of silver nitrate, the area of pores with the diameter higher than 1.4 nm is 5.21 m^2/g, and pores with the diameter of 1.57 nm are dominant, with the specific surface area of 0.76 m^2/g. In case of 5% of a silver nitrate additive, the area of pores with the diameter higher than 1.4 nm is 3.36 m^2/g, and pores with the diameter of 1.61 nm are dominant, with the specific surface area of 0.40 m^2/g.

An attractive technical solution is the application of composite core-shell nanofibers for the outworking of three-dimensional scaffolds that can combine antibacterial properties of the coating with bioactive properties of the inner core, for example, is the introduction of a medicine, or an antibiotic released after the dissolution of the coating. Such a structure can give an additional function of a carrier of a medicine to the outworked material. Those nanofibers are obtained by co-axial electrospinning using a flat collector. The selection of

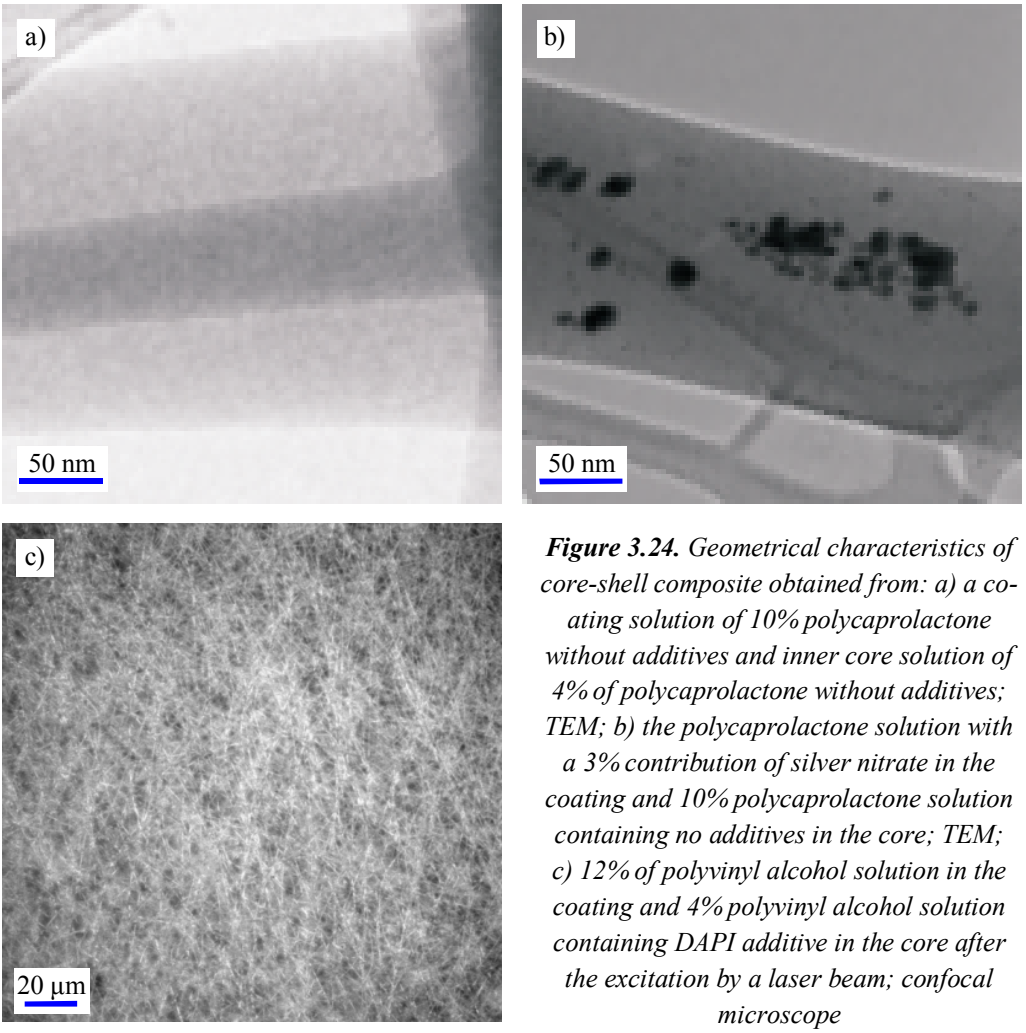

Figure 3.24. *Geometrical characteristics of core-shell composite obtained from: a) a coating solution of 10% polycaprolactone without additives and inner core solution of 4% of polycaprolactone without additives; TEM; b) the polycaprolactone solution with a 3% contribution of silver nitrate in the coating and 10% polycaprolactone solution containing no additives in the core; TEM; c) 12% of polyvinyl alcohol solution in the coating and 4% polyvinyl alcohol solution containing DAPI additive in the core after the excitation by a laser beam; confocal microscope*

materials which are components of the coating and the inner core of achieved fibers allows to design a tissue scaffold impacting on the body, including the elimination of microorganisms because of the presence of antibacterial silver in the coating of composite nanofibers, the disintegration of the coating and the unveiling of a bioactive core, supporting the development of tissues till the total rebuilt of appearing loss and the total decay of scaffolds to non-toxic products. The type and the properties of solutions used to manufacture core-shell nanofibers determine geometrical features and morphology, including the diameter of the produced fibers. Diversification of thickness of the inner core despite the continuous

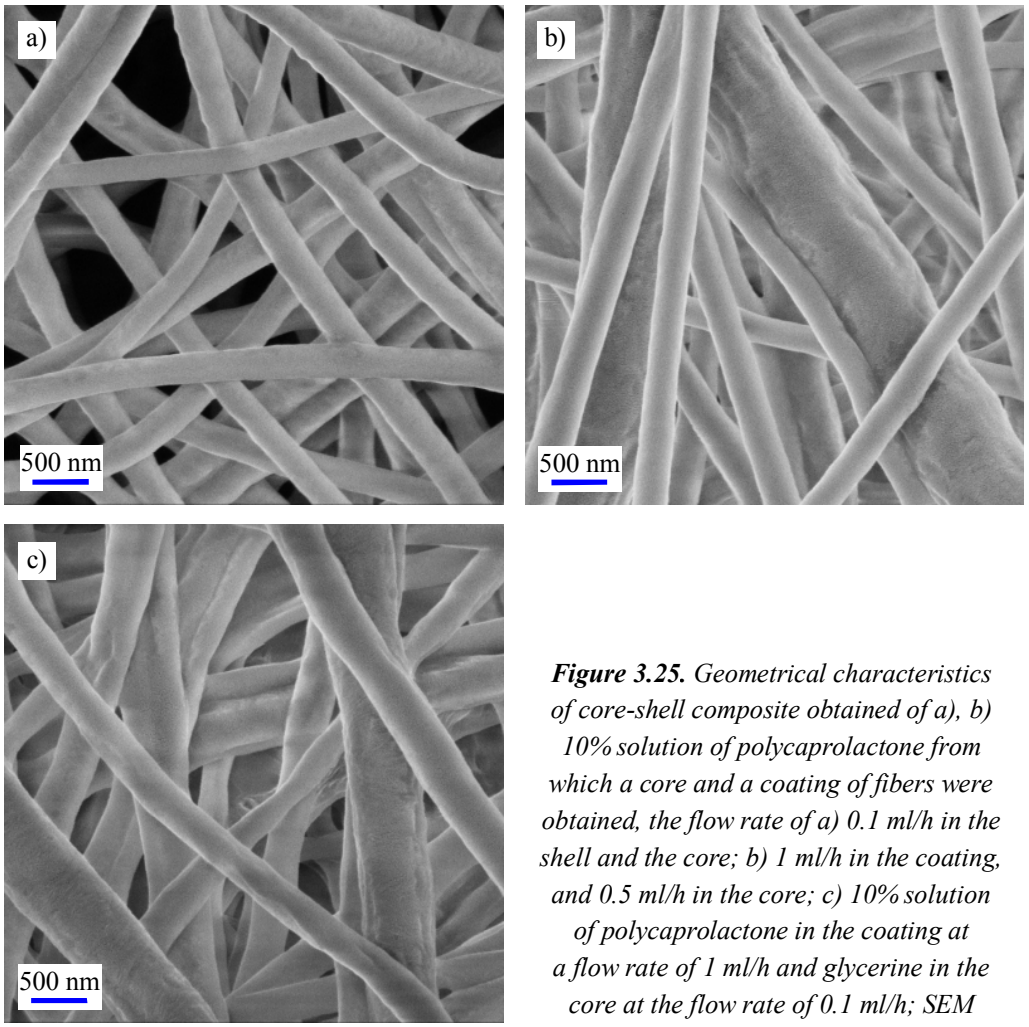

Figure 3.25. *Geometrical characteristics of core-shell composite obtained of a), b) 10% solution of polycaprolactone from which a core and a coating of fibers were obtained, the flow rate of a) 0.1 ml/h in the shell and the core; b) 1 ml/h in the coating, and 0.5 ml/h in the core; c) 10% solution of polycaprolactone in the coating at a flow rate of 1 ml/h and glycerine in the core at the flow rate of 0.1 ml/h; SEM*

flow of both solutions results from the use of solutions of different viscosity, including the coating solution of 10% polycaprolactone without additives, as the solution of as the inner core of 4% solution of polycaprolactone without additives (Fig. 3.24). In the case of the solutions significantly differing by electrical conductivity and viscosity, including in the coating solution of 3% polycaprolactone involving silver nitrate, and in the core of 10% polycaprolactone solution containing no additives, the fibers formed on the surface contains silver and have a diameter uniformity and shape. In the case of a core of a polycaprolactone solution containing 3% of silver nanoparticles in the coating 10% of polycaprolactone

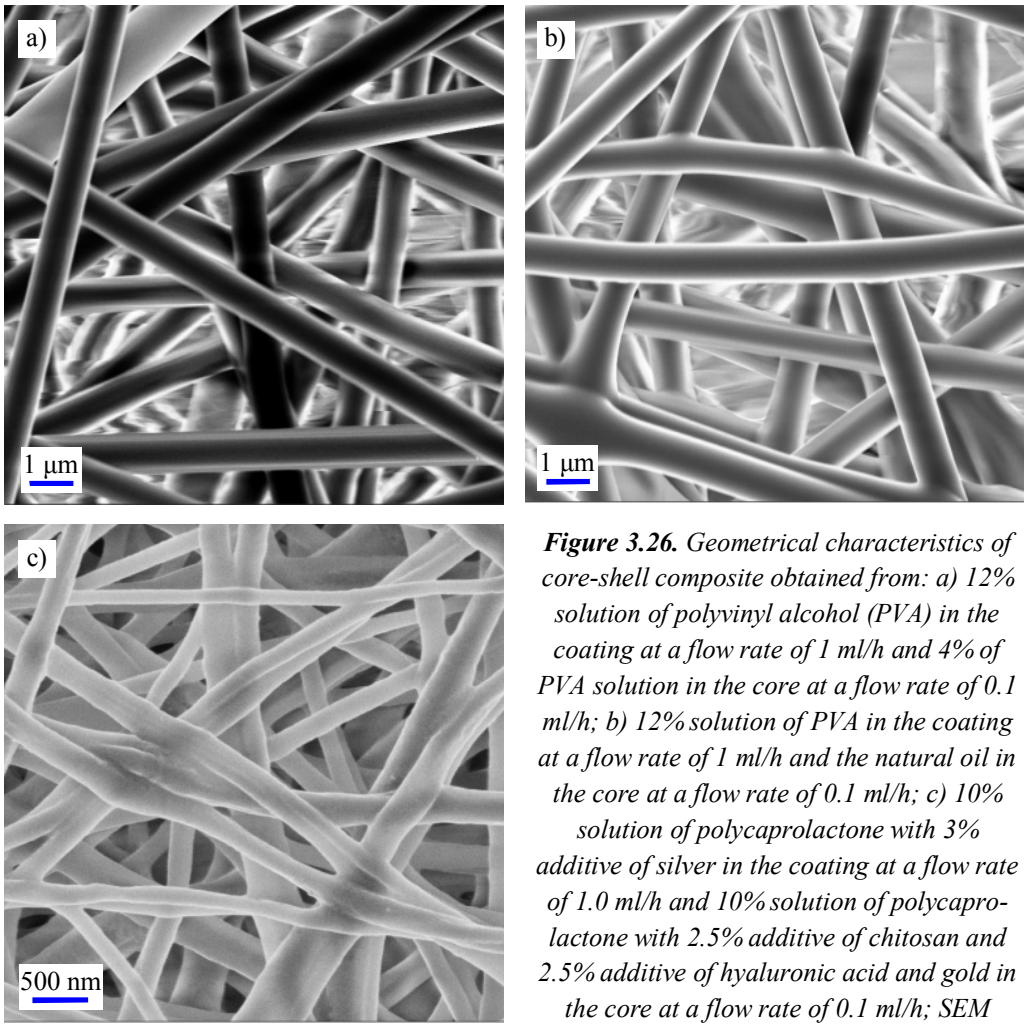

Figure 3.26. *Geometrical characteristics of core-shell composite obtained from: a) 12% solution of polyvinyl alcohol (PVA) in the coating at a flow rate of 1 ml/h and 4% of PVA solution in the core at a flow rate of 0.1 ml/h; b) 12% solution of PVA in the coating at a flow rate of 1 ml/h and the natural oil in the core at a flow rate of 0.1 ml/h; c) 10% solution of polycaprolactone with 3% additive of silver in the coating at a flow rate of 1.0 ml/h and 10% solution of polycaprolactone with 2.5% additive of chitosan and 2.5% additive of hyaluronic acid and gold in the core at a flow rate of 0.1 ml/h; SEM*

solution fibers with different geometric shapes and a tendency to agglomerate silver are formed (Fig. 3.24).

The structure of core-shell nanofibers was confirmed by scanning electron and transmission microscopy and as well by the use of confocal microscopy after the addition to the organic core of chemical compound DAPI (4',6-diamidino-2-phenylindole) as a fluorescent dye strongly binding to the acid deoxyribonucleic acid DNA on the principle of intercalation and commonly used to dyeing nuclei or chromosomes by visualization of DNA. In nanofibers coated with 12% solution of polyvinyl alcohol and a core of 4% polyvinyl alcohol solution, containing an

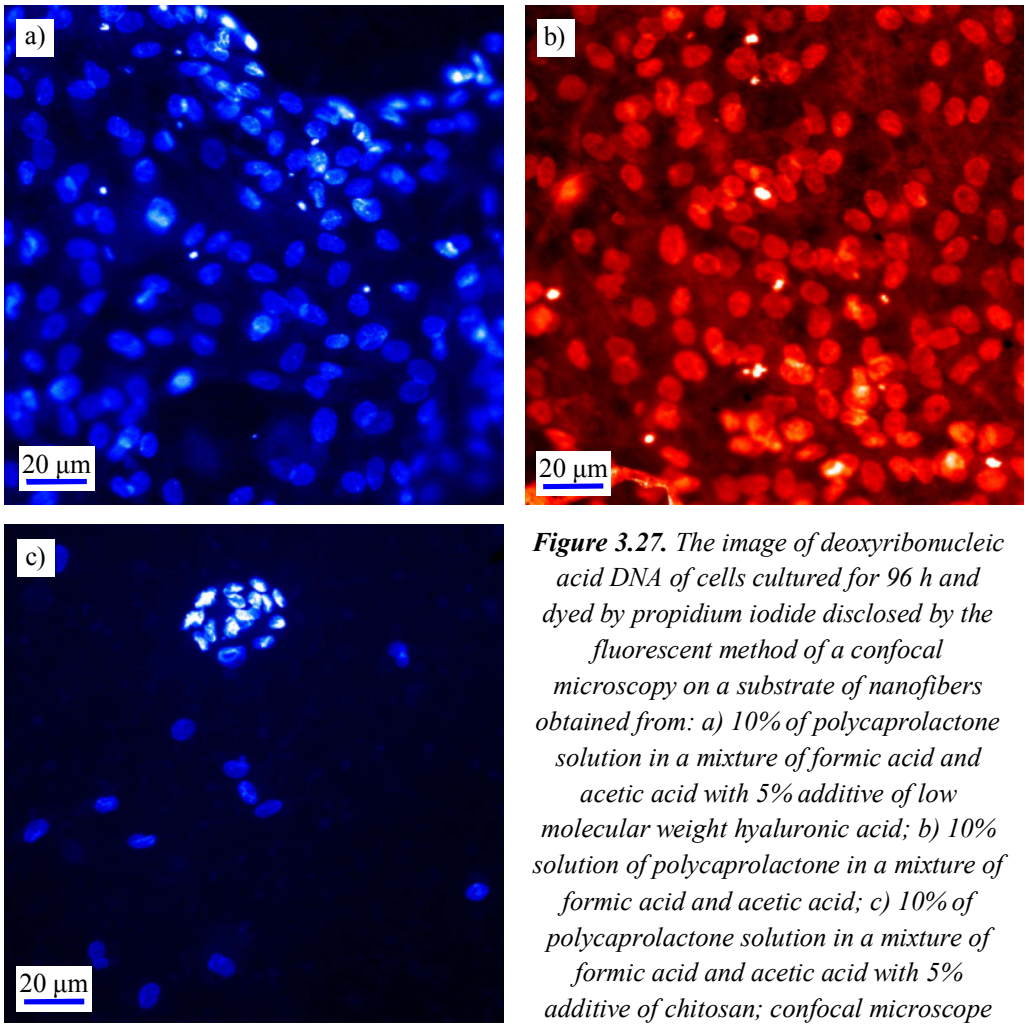

Figure 3.27. *The image of deoxyribonucleic acid DNA of cells cultured for 96 h and dyed by propidium iodide disclosed by the fluorescent method of a confocal microscopy on a substrate of nanofibers obtained from: a) 10% of polycaprolactone solution in a mixture of formic acid and acetic acid with 5% additive of low molecular weight hyaluronic acid; b) 10% solution of polycaprolactone in a mixture of formic acid and acetic acid; c) 10% of polycaprolactone solution in a mixture of formic acid and acetic acid with 5% additive of chitosan; confocal microscope*

additive DAPI as a result of which excitation by the laser beam in the confocal microscope followed by the illumination of fibers on the surface of a microscope glass (Fig. 3.24c).

Geometrical features and morphology of the produced fibers can be also adjusted by the flow rate of solutions. In the case of the application of 10% polycaprolactone solution, out of which the coating of the fiber core were achieved, at a flow rate of 0.1 ml/h a fiber diameter equals 200-600 nm, and at a flow rate of 0.5 ml/h is in the range of 200-1100 nm (Fig. 3.25) while in the case of the application of glycerine as a core solution at the flow rate of 0.1 and 0.5 ml/h, the diameter of the fibers is 200-1300 nm (Fig. 3.25c). The diameter of the fibers of

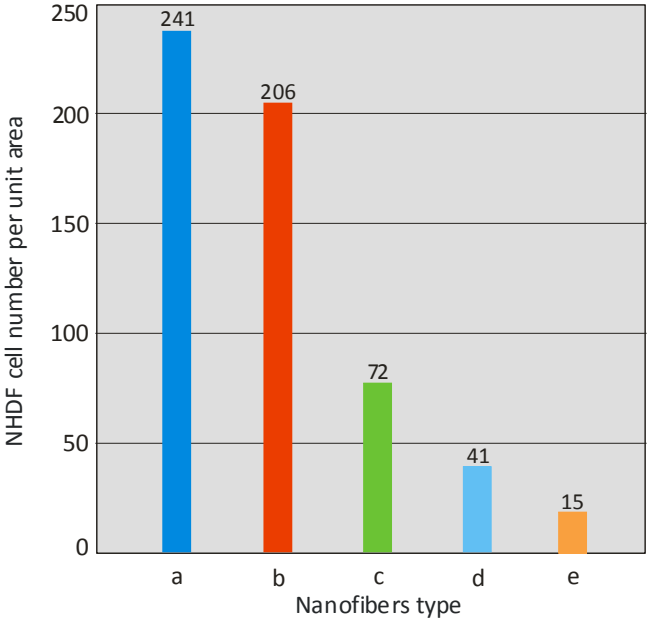

Figure 3.28. *The average surface density of deoxyribonucleic acid DNA of a cell cultured for 96 hours on substrate of nanofibers: a) are obtained with a 10% of polycaprolactone solution in a mixture of formic acid and acetic acid including the 5% additive of low molecular weight hyaluronic acid; b) obtained with 10% of polycaprolactone solution in a mixture of formic acid and acetic acid; c) comprising a mixture of 5% low molecular weight hyaluronic acid involving 2.5% contribution of chitosan and colloidal gold; d) containing 5% of chitosan; e) containing a mixture of 5% high molecular hyaluronic acid involving 2.5% contribution of chitosan, and colloidal*

the coating with the solutions containing 12% hydrophilic polyvinyl alcohol and the core of polyvinyl alcohol with 4% contribution is in the range of 700-1300 nm, whereas the core of the natural oil, the interval is in the range of 300-1200 nm (Fig. 3.26). Relevant differences in a diameter also show nanofibers prepared out of 10% polycaprolactone solution without additives, polycaprolactone containing 3% additive of silver nitrate which were used for the construction of the coating and 5% polycaprolactone solution without additives, polycaprolactone with 5% low molecular weight hyaluronic acid, polycaprolactone with 5% contribution of silver nanoparticles, polycaprolactone with 2.5% mixture of the additive of low molecular weight hyaluronic acid, 2.5% additive of chitosan and colloidal gold, used to prepare the solution of the inner core (Fig. 3.26c).

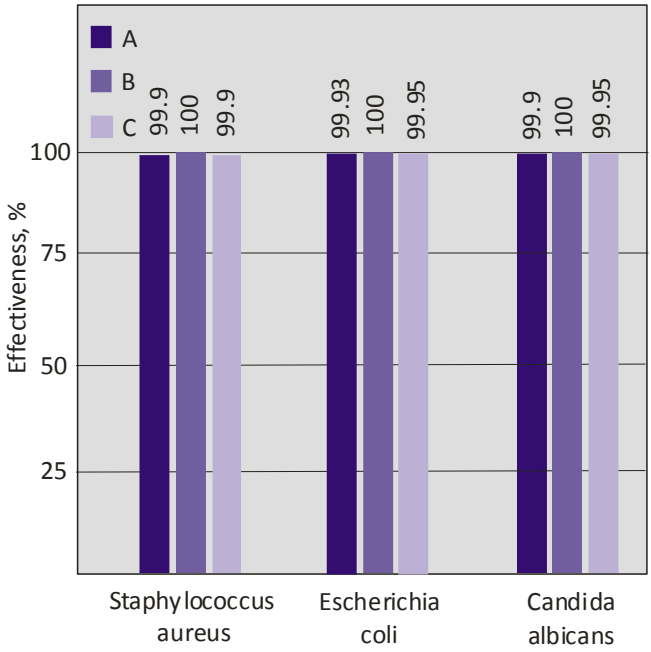

Figure 3.29. *Antibacterial efficiency (ABE) and antifungal efficacy (AFE) of double-component fibers obtained by dissolving PCL with the molecular mass of M_w = 70,000-90,000 g/mol obtained using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with AlphaSan additives with fraction of: A) 1%, B) 3%, C) 5%, after 17 hrs of incubation in the environment of microorganisms*

In order to evaluate the bioactivity of selected nanofibers, including the ones generated out of 10% polycaprolactone solution in a mixture of formic acid and acetic acid and nano-fibers containing, respectively, 5% of chitosan, 5% low molecular weight hyaluronic acid, a mixture of low molecular or high molecular hyaluronic acid with the participation of 2.5% chitosan with the participation of 2.5%, and colloidal gold, it caused that normal human dermal fibroblasts NHDF were grown on its substrate by 96 h and surface density of cells grown on different substrates, as the number referred to the unit area (Fig. 3.27). Rating of surface density of cultured cells were made using fluorescent confocal microscopy after dyeing with propidium iodide of deoxyribonucleic acid DNA of the cultured cells. Nanofibers 10% polycaprolactone solution in a mixture of formic acid and acetic acid comprising the additive of 5% low molecular weight hyaluronic acid (Fig. 3.28), slightly higher than the surface density of cultured cells NHDF 206 on nanofibers obtained with 10%

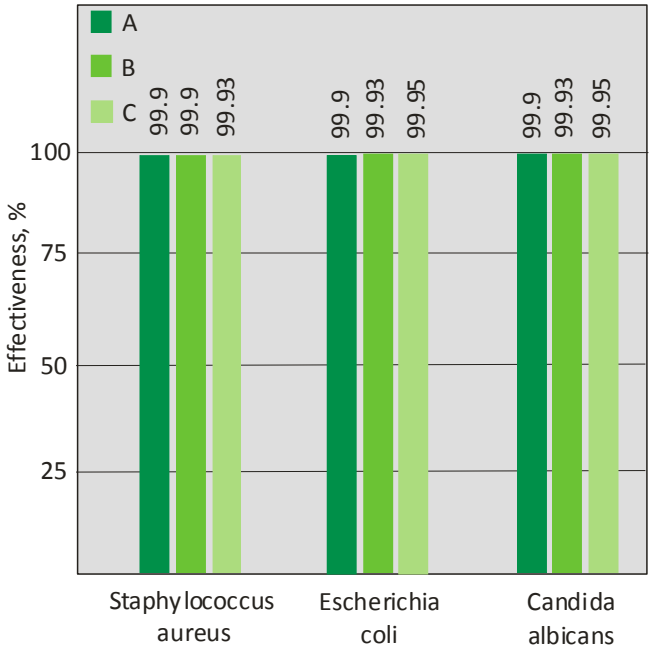

Figure 3.30. *Antibacterial efficiency (ABE) and antifungal efficacy (AFE) of double-component fibers obtained by dissolving PCL granulate with the molecular mass of M_w = 70,000-90,000 g/mol using 10% mixture of hydrochloric acid and formic acid with mass ratio of 70:30 with addition of $AgNO_3$ with fraction of: A) 1%, B) 3%, C) 5%, after precipitation of silver with 2% ascorbic acid solution and 17 hrs of incubation in the environment of microorganisms*

polycaprolactone solution in a mixture of formic acid and acetic acid have the highest average surface density of cultured cells NHDF 241. A significant difference in the effects of nanofibers containing low molecular weight and high molecular weight hyaluronic acid and weak bioactivity of nanofibers containing a combination of chitosan and polycaprolactone was shown.

The efficiency of the additives of silver nitrate, AlphaSan and chitosan introduced into double-component nanofibers in fighting Gram+ *Staphylococcus aureus* bacteria in anti-bacterialness examinations made in *in vitro* tests is diversified. The introduction of chitosan, regardless its fraction, does not improve antibacterial properties of the fibers obtained. The highest average antibacterial efficiency (ABE) of 99.93% in material groups containing 1 and 5% of the additive is recorded for nanofibers containing AlphaSan, while in the group with

3% of additives, the highest average antibacterial efficiency of 99.93% is characteristic for nanofibers containing AlphaSan (Fig. 3.29), and nitrate silver (Fig. 3.30).

There are also differences in how additives of silver nitrate, AlphaSan and chitosan with 1-5% fraction influence the antibacterialness of double-component nanofibers in *in vitro* tests on Gram- *Escherichia coli* bacteria. The highest antibacterial efficiency in relation to *Escherichia coli* bacteria for double-component nanofibers containing 1% of additive is indicated for nanofibers containing AlphaSan, analogously as in case of 5% of this additive, when it is 100%. The highest average antibacterial efficiency of 100% relative to *Escherichia coli* bacteria in a group of materials containing 3% of the additive is indicated for nanofibers containing 3% of silver nitrate and AlphaSan additive. Nanofibers containing 1-5% of chitosan do not show antibacterial properties in relation to *Escherichia coli* bacteria.

Double-component nanofibers containing 1-5% additives of chitosan do not exhibit antifugal properties, either, in an environment containing *Candida albicans* fungi. From among double-component nanofibers containing 1% of additive, the highest antifungal efficacy (AFE) in this environment of 99.9% is distinctive for nanofibers containing silver nitrate or AplhaSan. As the fraction of the additive is growing to 3%, the highest AFE in fighting fungi is exhibited by nanofibers containing AlphaSan. The highest AFE in a group of materials containing 5% of the additive is indicated for fibers containing AlphaSan.

3.4. Final remarks on fabrication and applicability of polymer nanofibers in regenerative medicine

This part of the work presents the outcomes of own investigations concerning the creation of long-resorbable composite nanofibers with a bioactive core and a bactericidal shell. The selection of the external shell components can be controlled through selection of shell thickness, application of a polymer with smaller molecular mass, by mixing with a polymer material with shorter resorption time. The applicability of polymer fibers in medicine depends on biocompatibility and non-toxicity of the material applied, which is influenced by the chemical purity of the materials applied and the toxicity of the input solvents. The potential toxicity of nanofibers should therefore be eliminated, starting with selection of materials used for obtaining solutions. Many other factors fundamental for the quality and properties of polymer nanofibers need to be taken into account to create single- and double-component nanofibers. Two solutions need to be used in co-axial electrospinning.

Solvents with moderate volatility, such as, e.g. a mixture of formic acid and hydrochloric acid, support the transformation of a solution under the influence of an electrostatic field in the form of a nanofiber, as their evaporation time equals the time necessary for its production. In case of solvents with considerable volatility, e.g. a mixture of chloroform and methanol or tetrahydrofuran and dimethyl sulfoxide, they support the fabrication of microfibers, as the time, in which solvents are evaporated, is shorter.

The type of the collector used may also influence the solvent evaporation rate. In the case where a collector is flat, the fibers tend to stick together, which is not the case if a rotating collector is used, in connection with the movement of the gas over the surface of such a collector, in particular as a result of synergic interaction of rotation motion of the cylinder and a gas flow system in the chamber. During electrospinning, the solvent is evaporated in the space between the electrodes, and solvent vapours coming from the solution constantly enter the gas situated between electrodes. In case of a flat collector, gas flow over its surface is insufficient to evacuate vapours of the solvents created between the flat collector and nozzle, even despite an installed system of evacuation of volatile products from a working chamber. For this reason the fibers stick together if a flat collector is used. The type of the collector used is also decisive for the spatial arrangement of fibers. The higher isotropy of fibers is obtained

by using flat collectors, whereas fibers anisotropy when using rotating collectors is increasing with the rotation speed of the collector.

The properties of the solutions in which nanofibers are manufactured, including viscosity and conductivity, are closely linked to the type and properties of solvents. The nanofibers obtained from a polymer material with higher molecular mass (70,000-90,000 g/mol) are thinner as compared to those of the fibers obtained using polymer with considerably smaller molecular mass (45,000 g/mol). If electrostatic voltage is indeed applied in the both mentioned solutions, a Taylor cone is created, however, it is more stable in case of a solution with higher molecular mass as the interactions between macroparticles are increased. This, on the other hand, translates into the intensification of friction forces created between macroparticles and is supportive to the fabrication of fibers with their diameter similar to each other and to a decreased number of defects such as beads on the surface of fibers. The opposite situation takes place when the molecular mass of polymers is decreased. The destabilisation of a Taylor cone with the shorter length of a macroparticles chain is conducive to the formation of fibers with different diameter.

The diameter of the fibers obtained may change after introducing additives, e.g. silver nitrate and chitosan, which are enhancing electric conductivity. In the first case it is related to the presence of an atom of silver in a silver nitrate particle, while in the second case with the presence of polar chemical groups chitosan is made of, and which includes -acetamido-2-deoxy-β-D-glucopyranose particles and 2-amino-2-deoxy-β-glucopyranose groups containing, notably, an atom of nitrogen. Such groups support the formation of hydrogen bonds, thus impacting the change of electric conductivity of the solutions obtained. When AlphaSan is introduced into a solution, despite containing 10% of silver, it reduces the electric conductivity of solutions. By introducing any of the additives, the viscosity of solutions is increased due to a higher density of the solution achieved. The higher viscosity of the solutions obtained related to the activity between macroparticles, does not translate into smaller diameter of the fibers obtained. Such additives are impacting, however, the BET, Langmuir specific surface area and the area of pores. If chitosan is introduced, the specific surface area is decreased related to a larger diameter of the fibers produced. The opposite tendency occurs when the fraction of AlphaSan and silver nitrate is increased, leading to an increase in the specific surface area of fibers, as agglomerates of AlphaSan and silver crystals on the surface of fibers exist. The

additives introduced have also substantial influence on the antibacterialness and antifungalness of nanofibers. Silver nitrate and AlphaSan show high efficacy in fighting Gram+, Gram- bacteria and fungi, and macromolecular chitosan does not show antibacterial and antifungal properties. The additives mentioned are influencing the bioactive properties differently, by interacting with the cells of NHDF fibroblasts. The highest bioactivity is characteristic for nanofibers containing an additive of low molecular hyaluronic acid, whilst macromolecular hyaluronic acid is of much lesser importance.

The analysis made clearly shows a clear development trend of all the world markets of biomaterials and various medical products, and projections are very promising. This is mainly related to the growing population of people at the retirement age and the ageing of the society, increased healthcare spendings, development of state-of-the-art medical technologies and diagnostic methods of many disorders, a higher incidence of different diseases, including civilisational diseases, with the dramatic growth of bodily injuries as a result traffic accidents and the related practising of sports. The resulting injuries, especially post-accident defects, post-resection defects and such connected with operative treatment of cancerous tumours, inflammation processes and other disorders of organs or tissues, make it necessary to replace or supplement such organs or tissues, to replace the functions of the lost tissue, with influencing actively the tissue environment, as well as to sustain and accelerate the naturally occurring regenerative processes to restore patients' vital functions. The circumstances listed above create multiple challenges to the scientific, not only medical environment, but also to tissue engineering and material engineering and manufacturing engineering to present newer and newer technical solutions enabling prosthetics, especially implantation. Polymer nanofibers create significant applicational possibilities in this field. The outcomes of own investigations presented in this part of the work are serving this purpose. The composite materials obtained, due to their non-toxicity resulting from the components applied, including solvents, bacteriocidity and bioactivity, may find their applications in tissue engineering as membranes in controlled regeneration of bone tissue, as carriers of medicinal agents in bone surgery, as implantable surgical meshes and as scaffolds for a tissue culture.

In turn, the composite core-shell nanofibers, by combining the antibacterial properties of the coating with bioactive properties of the core, are attractive materials for three-dimensional tissue scaffold. Such materials can be used as a carrier of medicine, a treatment of hard healing

wounds, invasive surgery, neurosurgery, as a substrate for the culturing of a retina, material to reconstruct nerves and in dentistry or oncology, to replace the natural tissue removed because of a cancer with the possibility of applying a therapeutic agent, e.g., an antibiotic or a medicine used in cancer therapies, released after the dissolution of the coating of nanofibers.

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