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Research article

Preparation and characterization of novel nanofibrous composites prepared by electrospinning as potential nerve guidance conduits (NGCs)

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Abstract. Appropriate protection and guiding are crucial during peripheral nerves repair. New generation nerve guidance conduits (NGCs) should not only provide mechanical support for the damaged nerve but also support healing processes. One of the most promising tissue regeneration applications is fibrous biomaterials since they are characterized by high porosity, flexibility, and strength. Additionally, they enable cell adhesion and proliferation. In this study, novel fibrous nanocomposites were obtained by applying the electrospinning technique, using polylactic acid (PLA) as a polymeric matrix which was further modified with metallic nanoparticles coated with conductive polymers. Such an approach resulted in the obtainment of biomaterials with a potential ability to conduct nerve impulses. The chemical structure of the obtained composites, as well as the morphology of ready products and separate nanocomponents, were investigated using Fourier-transform infrared spectroscopy (FTIR), transmission electron microscope (TEM) and scanning electron microscope (SEM) techniques. Furthermore, conductive and swelling properties in various media were determined. Finally, biomaterials were confirmed to be non-cytotoxic to L929 mouse fibroblasts and 1321N1 human glial cells. Based on the presented results, it can be concluded that nanofibrous nerve guidance conduits have all the key properties in the process of peripheral nerve regeneration and may constitute an important step in novel NGCs development.

Keywords: *material design and synthesis, biomaterials, nanofibers, nerve guidance conduits, tissue engineering*

1. Introduction

The nervous system is considered as the most important of the systems in the human body since it controls the work of other systems. The basic division of the nervous system includes the central nervous system and the peripheral nervous system. The peripheral nervous system, unlike the central nervous one, can regenerate [1]. Peripheral nervous system injuries occur most often during car accidents, at work in high-risk positions, during sports, and due to iatrogenic errors. Peripheral nerve damage can also be the result of chronic disease. The classification of nerve injuries based on the degree of damage was first introduced in 1943 by Seddon [2]. He distinguished the following three forms of injury: *neuropraxia*, *axonotmesia* and *neurotmesia*. The mildest form is neuropraxia, which is distinguished by the absence of axonal damage and segmental demyelination and results in weakening or blocking of nerve conduction. The symptoms include disturbance of sensation and motor function of the muscles. However, they disappear after a relatively short period of

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time, usually several days. In the case of *axonotmesia*, the axons are damaged without demyelination, thanks to which the continuity and integrity of the tissue are maintained. The nerve functions disappear, and there is no possibility of regeneration. Such a condition may last from several to dozens of months. The most severe form of injury is neurotensin, which is associated with a complete interruption of nerve continuity. This injury requires surgical intervention as there is no possibility of spontaneous regeneration [2].

Seddon's classification in 1951 was extended by Sunderland, who distinguished five degrees of nerve damage. Stage I and V correspond respectively to neuropraxia and neurotmesis according to the Seddon classification. Grades II to IV, on the other hand, correspond to *axonotmesia*, but distinguish connective tissue damage. In stage I, no damage to the perineural tissue is observed. In stage III, the endoneurium is damaged without changes in the *epinerium* and *perineurnum*. Stage IV involves damage to all sheaths except the epineurium [3]. In the case of injuries requiring surgical intervention, autologous transplantation is considered to be a "gold standard", however, it is associated with many limitations, including insufficient tissue availability, additional burden of the body associated with the procedure at the donor site, and the risk of neuroma. Another solution is allogeneic transplantation, but it also has some downfalls, such as the risk of rejection of the transplanted tissue, and it is necessary to use immunosuppressive drugs. In addition, prior tissue decellularization is required. A solution that is not associated with the problematic issues listed above methods is the use of synthetic nerve guidance conduits. The first attempts involved implanting a different type of tissue, such as a decalcified bone, artery, or muscle, at the site of nerve tissue damage. However, due to poor results and many disadvantages, it was necessary to carry out an attempt was made to fabricate nerve channels from synthetic materials. The first-generation nerve cords were made of silicone and polytetrafluoroethylene. They provided good mechanical support, but because they were not resorbable, thus another operation was required to remove the material. Noteworthy, these channels formed a barrier preventing the influx of growth factors, nutrients, and oxygen. Second-generation nerve cords are porous, biodegradable tubes made of well-studied, biocompatible materials such as polyglycolic acid (PGA) and poly-DL-lactide-*co*-caprolactone (PLCL), type I collagen or fibroin [4].

Some of the first- and second-generation nerve conduits have undergone in vitro and in vivo tests and have been approved by the Food and Drug Administration (FDA) for use [5–7]. Due to limitations, such as supporting the regeneration of only short nerve segments of up to three centimeters and lower efficacy compared to autografts, research into the third generation of nerve ducts is ongoing. In the third generation, the nerve guidance conduits are designed not only to provide mechanical support to the rebuilding tissue but also to stimulate tissue regeneration through a special topography, delivery of neurotrophic factors, stem cells or Schwann cells, extracellular matrix proteins, controlled release of drugs or ability to conduct electrical impulses. To achieve a biological effect, it is necessary to provide impulses of appropriate voltage delivery to the cultured cells. At the same time, the biomaterial provides mechanical support and does not undergo structural or chemical degradation as a consequence of electrical field impact. Importantly, NGCs should maintain their stability and exhibit a correlation between biodegradation rate and injured nerve recovery to prevent unmature tissue exposure to internal factors [5].

Electrospinning is a process enabling the production of micro or nanofibers from polymer solutions using an electric field. The main elements of the electrospinning device are a tank for a polymer solution, a power supply constituting a high-voltage source, a spinneret, and a grounded collector [8–11]. Most often, a metal needle is used as a spinneret, acting as an electrode. As a result of applying an electric voltage in the polymer solution, electric charges are generated. This leads to a change in the shape of the liquid droplet into a Taylor cone. The solvent is then evaporated, and the electrically charged stream is drawn towards the collector [12]. The electrospinning process is influenced by parameters such as the average molar mass of the polymer and its degree of branching, the amount of electric voltage, the molecular weight of the polymer, the properties of the solution (viscosity, conductivity, and surface tension), the distance between the needle and the collector, environmental parameters (temperature, humidity, and chamber airflow), manifold movement and size, *etc*. [6].

Nanofibrous biomaterials are characterized by a large specific surface area, high porosity [13], low density, and mechanical strength [9]. Therefore, they have similar structural features and functions to the extracellular matrix (ECM) [10, 11]. Moreover, the addition of the right type of nanoparticles in tissue engineering can significantly improve the biological, mechanical, and electrical properties of the scaffolds and can also provide various functions depending on the applications [8]. Nanoparticles also increase the rate of cell proliferation and affect the differentiation of mesenchymal stem cells. Nanocomposites show better mechanical properties compared to scaffolds not reinforced with nanoparticles [13–15]. This is due to the formation of new bonds (mainly hydrogen ones) between the nanoparticles and the polymer matrix. Due to the risk of infection, it is particularly important in tissue engineering to prevent microbial contamination of the substrates used. Some metal nanoparticles have antimicrobial activity; for example, silver nanoparticles have been shown to be able to fight some antibiotic-resistant bacteria [14]. Conductive polymers such as polyaniline (PANI), polypyrrole (PPy) and polyvinylpyrrolidone (PVP), among others, are widely used in biomedical applications [16–20] due to their biocompatibility and unique electrical and optical properties, like inorganic semiconductors and metals [18–24]. An additional advantage of conductive polymers is easy and cheap synthesis, as well as the possibility of modification [25–34].

Polylactic acid (PLA) is one of the most widely used biopolymers in various industries. Its physical, mechanical and thermal properties are related to its mass stereochemistry and molecular distribution so that it exists in an amorphous or semi-crystalline state. Due to its biocompatibility, biocompatibility and biodegradability, it has been approved by the FDA for direct contact with biological fluids. The production of PLA is environmentally friendly as it requires 25–55% less energy than the production of gasoline-based polymers, the raw materials can be derived from renewable resources and it is recyclable. PLA moldability allows it to be processed by various techniques such as extrusion, thermoforming, injection molding, blow molding, and electrospinning into any form, depending on the application. The disadvantages of PLA include its hardness and brittleness, but the use of nanoparticles and processing into the form of nanofibers can significantly improve these parameters. In addition, the hydrophobicity of PLA reduces the induction of cellular processes and surface interaction with proteins and cells so that the material does not have the ability to integrate with the surrounding environment, which can cause an inflammatory reaction. Other disadvantages of PLA include the lack of reactive groups in the side chains, which makes it chemically inert, making it difficult to modify [35–37].

Electrospun PLA fibers are similar to the extracellular matrix and have a large specific surface area and high porosity. Depending on the application, the morphology of the fibers, and thus the mechanical, physical and biological properties of the structure of the biomaterial, can be changed by changing the electrospinning parameters. The most important factors influencing the electrospinning of PLA include the polymer concentration, the molecular weight of the polymer (M_w), and the type of solvent or solvent system. Studies have shown that PLA with a higher M_w shows greater elongation at break due to greater entanglement of the chain. A higher concentration of the solution increases viscosity, which leads to reduced stretching of the fibers and, consequently, to the formation of thicker fibers. If the concentration and M_w are too low, droplets instead of fibers are formed due to the low viscosity of the solution [38–40].

Another factor influencing the electrospinning process is the selection of solvents. When producing nanofibres for tissue engineering applications, it is important that the dissolvers used are non-toxic. The addition of dioxane to acetone as a cosolvent affects the faster dissolution of PLA, stabilization of the electrospinning process and obtaining fibers without defects in the form of pearls [40–42].

The aim of this research was to develop a new type of biomaterial that would act as a nerve guide conduit for a third generation. The goal was achieved by using electrospinning to produce porous 2D mats modified with metallic nanoparticles coated with conductive polymers to increase their mechanical properties and biofunctionality. Our study revealed that newly obtained potential scaffolds for nerve tissue regeneration are nontoxic to two different cell lines and positively affect cell proliferation, which shows their potential in nerve injury treatment.

2. Materials and methods

2.1. Materials

Poly(L-lactic acid) (PLA) of molecular weight 193.3 kg/mol was purchased from Good Fellow (UK). Acetone, dioxane, tetrahydrofurane (THF), *N*-methylpyrrolidone warch, silver nitrate, chloroauric acid, chloroplatinic acid, polyaniline, polypyrrole, The European Collection of Authenticated Cell Cultures (ECACC), Dulbecco's Modified Eagle Medium (DMEM) with glucose content cell culture medium, fetal bovine serum (FBS), phosphate buffer solution (PBS), $CaCl_2 \cdot 2H_2O$, $MgSO_4$, KCl, KOH, KH_2PO_4, Na_2HPO_4, $NaHCO_3$, NaCl, HCl, NaOH, mouse fibroblasts L929 cell line and 1321N1 Cell Line human glial cells for commercial use, trypsin with EDTA, antibiotics (streptomycin/penicillin) were purchased from Sigma Aldrich, Poznań, Poland.

2.2. Methods

2.2.1. Preparation of conductive nanoparticles (NPs)

Gold, silver, and platinum NPs were obtained by reducing silver nitrate, chloroauric acid and chloroplatinic acid. The concentrations of the solutions were $AgNO_3$ 197 mg/250 cm^3 H_2O,
$HAuCl_4 \cdot 3H_2O$ 215 mg/250 cm^3 H_2O,
$H_2(PtCl_6) \cdot 6H_2O$ 312.5 mg/250 cm^3 H_2O.
Extracts of freeze-dried ginger, orange peel, lawsonia, hawthorn fruit, dandelion root, and peppermint were used to reduce the nanoparticles, which were prepared by 24 h extraction of 10 g of plant material with ethanol, and then the extracts were diluted to a volume of 200 cm^3. To investigate possible antioxidant properties, the content of phenolic and polyphenolic compounds in the obtained extracts was determined by the Folin-Ciocâlteu method. To investigate the ability of free radical removals 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) tests were carried out according to Next, 2 cm^3 of plant extract and 4 cm^3 of water were added to 4 cm^3 of noble metal ion solutions. Syntheses were carried out according to variable parameters given in Table 1 in a microwave reactor. Next, the nanoparticles were purified from unreacted substrates using dialysis tubes. To coat the surface metallic nanoparticles with conductive polymers, 2 cm^3 of PANI (50 mg/10 cm^3 THF) or 4 cm^3 of PPy (60 mg/10 cm^3) were added to the intensively miscible solutions (10 cm^3). Increasing the amount of added polymer resulted in the agglomeration of nanoparticles.

Table 1. Investigated parameters of ecofriendly NPs synthesis.

Parameter	Range
Temperature	60–100 °C
Reaction time	10–30 min
MW power	10–25%
Extract concentration	5–50%

2.2.2. Preparation of NPs/PLA nanofibers nanocomposites

A 10% PLA solution was prepared by dissolving PLA in a mixture of 90% acetone and 10% dioxane solvent mixture. The nanoparticles were centrifuged, dried and then dispersed in an ultrasonic cleaner with the addition of acetone (Table 2). Nanofibers were obtained using a voltage of 25.5 kV, the distance of the metallic needle (surgical steel) from the collector was set to 5 cm, and a flow rate of NPs/PLA solution was 0.15 ml/min.

2.3. Physicochemical properties study of NPs/PLA nanofibers nanocomposites and components

2.3.1. Chemical structure study

The chemical structure was evaluated using Fourier-transform infrared spectroscopy (FT-IR) equipped with an ATR adapter. For the experiments, Thermo Nicolet Nexus 470 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used.

2.3.2. Porosity and density measurements

Tested samples were placed into the measured volume of isopropanol. After 5 min the change in volume of the alcohol-impregnated nanomaterials was determined. Then, the investigated samples were removed from the isopropanol. After that, the difference in alcohol volume was measured. The obtained

Table 2. Chemical composition of prepared composites.

Sample	Polymer	Nanoparticles
1.	PLA	–
2.		Au
3.		Ag
4.		Pt
5.		Au/PP
6.		Ag/PP
7.		Pt/PP
8.		Au/PANI
9.		Ag/PANI
10.		Pt/PANI

data were used to calculate the density (Equation (1)) and porosity (Equation (2)):

$$d = \frac{W}{V_2 - V_1} \tag{1}$$

$$p = \frac{V_1 - V_3}{V_2 - V_3} \cdot 100\% \tag{2}$$

where d is the density [g/cm^3], p is the porosity [%], W is the weight of the investigated sample [g], V_1 is the initial volume of isopropanol [cm^3], V_2 is the volume of isopropanol with immersed sample [cm^3], V_3 is the volume of isopropanol after sample removal [cm^3].

2.3.3. Determination of swelling degree in water, SBF and PBS

To determine the swelling abilities of the prepared nanofibers, 10 mg of each sample was placed in distilled water, simulated body fluid (SBF) (pH = 5.5) and phosphate-buffered saline (PBS) and left for 24 h. After fixed period intervals, the sample was weighed again. The swelling degree was calculated using Equation (3):

$$SD = \frac{W_t}{W_0} \tag{3}$$

where SD is the swelling degree [g/g], W_t is the sample weight after 24 h [g], W_0 is the initial weight of the sample [g].

2.3.4. Conductivity study

The conductivity of purified nanoparticle aqueous solutions was measured using the ELMETRON CC-411 conductivity meter. The conductivity of the ready membranes was determined using ELMETRON CX-742 using graphite electrodes with a surface area of 1 cm^2. Materials with a thickness of 0.1 mm have previously been swollen in PBS solution. Tests were carried out at room temperature.

2.3.5 Mechanical properties study

To verify the mechanical durability of the prepared membranes, Tensile strength was determined. For this purpose, membranes were cut into dog bone shapes (dimensions – thickness 1.0 mm and overall length 150 mm, measuring part width 10 mm). Tests were carried out according to N-EN ISO 527:1998 standard for plastics mechanical properties evaluation.

2.4. TEM analysis

Nanomaterials were investigated by a transmission electron microscope purchased from Jeol, (Peabody, MA, USA).

2.5. SEM analysis

Ready products were investigated by FEI Quanta 650 FEG scanning electron microscope purchased from FEI (ThermoFisher Scientific, Oregon, USA). Samples before analysis were sputtered with copper.

2.6. Cytotoxicity study

The observation of cell morphology under the inverted microscope was performed using 40× and 100× magnification (Delta Optical IB-100 microscope, Planeta Oczu, Zielona Góra, Poland). For the experiments, and 1321N1 Cell Line human glial cell was used and the L929 cell line, which is commonly applied for biomaterials cytotoxicity study according to ISO 10993 norm. The culture was conducted for 72 h under standard conditions (5% CO_2 concentration, high humidity, 37 °C) using DMEM (Gibco) as a medium which was changed every 48 h.

3. Results and discussion

To prepare nanofibers, an electrospinning system was constructed consisting of a syringe connected to a pump that enables precise, uniform pumping of the polymer solution, a PP pipe, metallic needle and an aluminum cylinder, which was used as a collector. Both elements were connected to a high-voltage DC power supply. During electrospinning, the roller was rotated at a speed of 1000 rpm. Introducing the roller into a rotational motion resulted in the evenly settling of fibers on the entire surface of the collector and obtaining its small diameter (Figure 1).

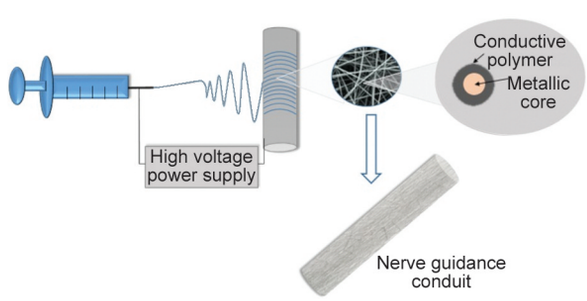

Figure 1. General scheme of obtaining nerve guidance conduits.

3.1. NPs characteristics

To determine the potential biological activity of the nanomaterials in terms of possible antioxidant activity, all extracts were evaluated over the content of phenolic and polyphenolic compounds. It can be noticed that the highest content was obtained for Lawsonia and peppermint, while the lowest was for dandelion root and orange peel. As shown in Table 3, the amount of phenol compounds was correlated with the ability to remove free radicals. As it can be observed, in Table 4 extracts from lawsonia and peppermint were able to scavenge free radicals in almost 100%. Another important parameter was the ability of the extract to take part in silver, gold and platinum NPs obtainment, which was successful in each case, yet with different effectiveness. Microwave-assisted, green synthesis of metal NPs using ginger extract as a reducing agent resulted in the obtainment of the product with the highest efficacy, as shown in Table 5. TEM analysis confirmed the formation of nanoparticles with a size in the range from 10 to 100 nm. Natural extracts-mediated NPs green synthesis is a facile and promising alternative to traditional synthesis routes since it enables the preparation of nanoparticles with great stability and high biocompatibility. The reduction process of metal ions occurs due to the presence of such natural compounds like isoflavones, gallic or protocatechuic acid. As shown in Figure 2, prepared nanoparticles are characterized by a uniform, rounded shape and small-size dispersion. Such morphology is desired for biomedical applications since it prevents bioaccumulation in organs and should not cause any cellular damage due to sharp edges.

The analysis also confirmed the coating of the nanoparticles with conductive polymers (Figure 3). In all cases, a successful surface modification of NPs can be noticed. However, some differences can be spotted

Table 3. The content of phenolic and polyphenolic compounds in the obtained extracts was determined by the Folin-Ciocâlteu method.

	Extract	Phenol compounds content [mg/ml]
1.	Ginger	7.47
2.	Orange peel	5.04
3.	Lawsonia	13.17
4.	Hawthorn fruit	10.75
5.	Dandelion root	3.71
6.	Peppermint	11.01

Table 4. Antioxidant properties of extracts.

	Extract	DPPH [%]	ABTS [%]
1.	Ginger	75.7	42.9
2.	Orange peel	51.8	29.5
3.	Lawsonia	98.5	90.3
4.	Hawthorn fruit	72.7	31.3
5.	Dandelion root	52.2	6.3
6.	Peppermint	95.2	53.5

Table 5. Metal nanoparticle synthesis efficiency depending on the extract type.

	Extract	Nanoparticle synthesis efficiency [%]		
		Gold	Silver	Platinum
1.	Ginger	100	100	93
2.	Orange peel	100	97	90
3.	Lawsonia	97	82	81
4.	Hawthorn fruit	100	93	89
5.	Dandelion root	91	90	81
6.	Peppermint	95	95	87

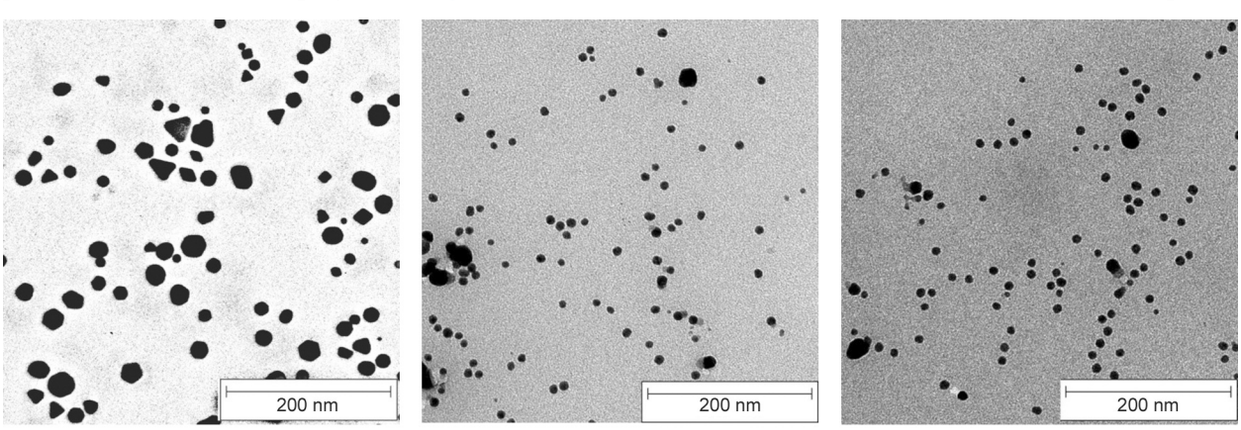

Figure 2. TEM microphotographs of the nanoparticles: a) Au; b) Ag; c) Pt.

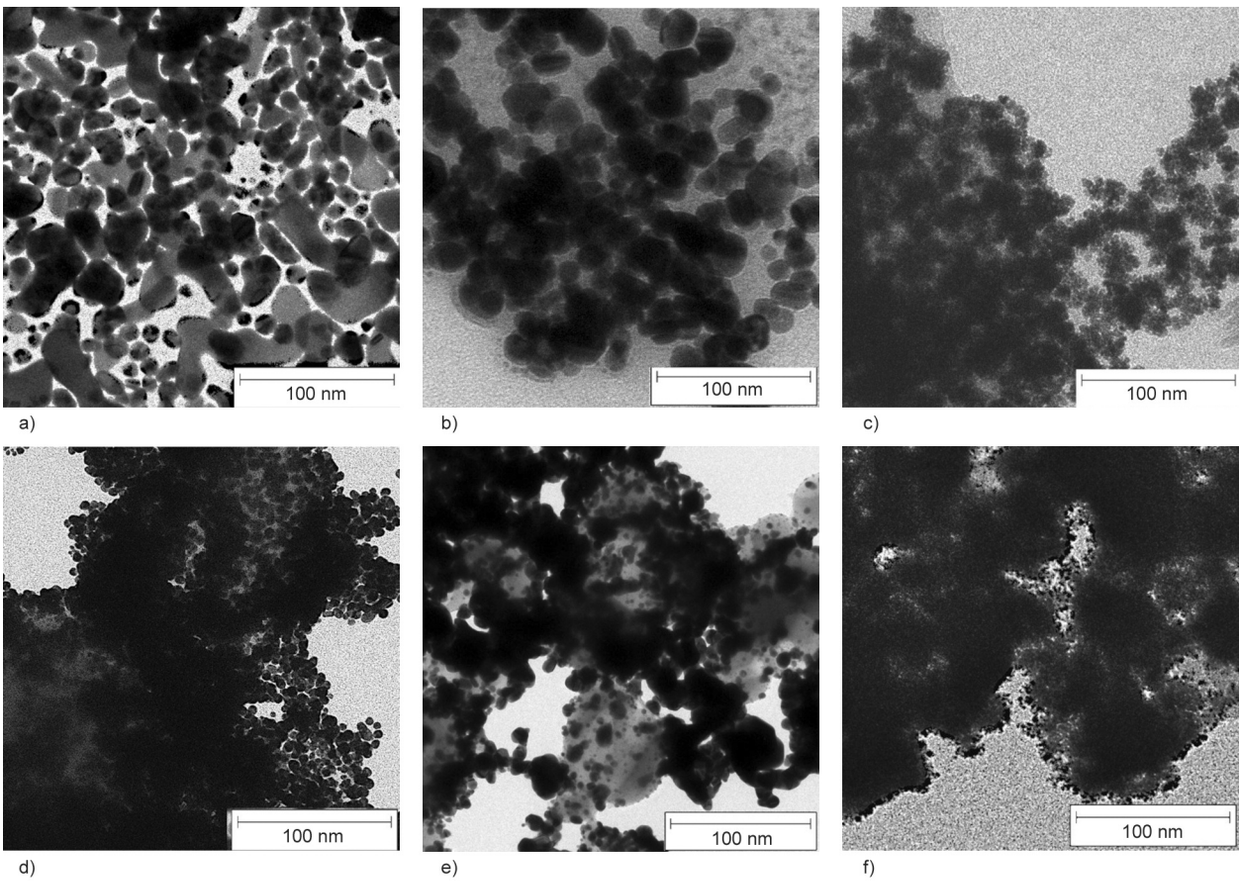

Figure 3. TEM microphotographs of the nanoparticles: a) Au/PP; b) Ag/PP; c) Pt/PP; d) Au/PANI; e) Ag/PANI; f) Pt/PANI.

between the two polymers. In the case of PP, all kinds of NPs are uniformly coated, and single nanoparticles can be distinguished. A small size increase of the particles can be noticed due to the polymers' presence; however, materials are still in size below 100 nm in at least one dimension. On the contrary, using PANI for NPs modification increased their tendency for agglomeration, which potentially may have a negative impact on their both biological and physical parameters, typical for the nanoscale objects. No significant changes between types of nanoparticles can be observed, which suggests that the proposed strategy for NPs modification with conductive polymers can be said to be versatile.

3.2. FT-IR chemical structure analysis

The first step in advance of NGC preparation was eco-friendly conductive hybrid nanoparticle preparation. To enhance metallic (Ag, Au, Pt) NPs biofunctionality and integration with polymeric matrix, nanoparticles coating with polypyrrole (PP) and polyaniline (PANI) was performed. The major role of this combination was to provide the possibility of cell electrostimulation during nerve injury therapy using NGCs since this method is known to enhance and accelerate nerve tissue formation and biological functions of damaged regions restoration.

To determine the purity of the obtained nanofibers, the ATR/FTIR spectrum was collected (Figure 4). PLA is a polymer characterized by a simple chemical structure; it is obtained as a result of a ring-opening polycondensation reaction. The spectrum shows two bands at 2996 and 2946 cm^{-1} coming from the chemical bonds of the C–C–H polymer chain. The typical band at 1752 cm^{-1} confirms the presence of an ester group. The spectrum above 3000 cm^{-1} does not show any bands originating from –OH groups, which proves the high molecular weight of the. In the spectrum, other chemical bands cannot be observed in the range of 1690–740 cm^{-1}, which indicates the absence of carboxyl, ketone and aldehyde groups. It proves the high purity of the tested nanofibers. Due to the relatively low content of the NPs, no bands typical for metallic nanoparticles were achieved during each spectrum collection or polymeric (PP, PANI) coatings [25–27].

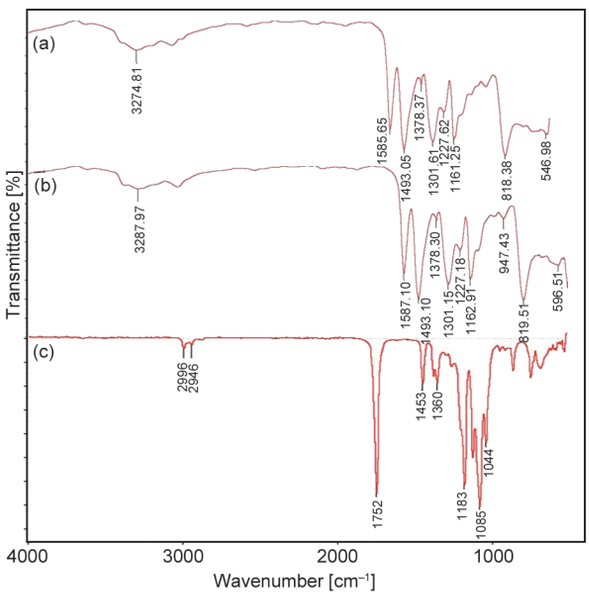

Figure 4. FT-IR spectrum of the: a) Au nanoparticles obtained using ginger extract coated with PP of the nanofibers; b) Au/PP Au nanoparticles obtained using ginger extract coated with PANI; c) nanofibers.

3.3. Porosity and density

Obtained nanocomposites were characterized by high porosity and low density, characteristic of nanofiber biomaterials. A large number of pores is a highly demanded product feature when dedicated to cell culture applications since it provides the possibility of oxygen, nutrients and signaling biomolecule delivery at the same time, giving a possibility of cell migration and proliferation in 3 dimensions. It also enhances extracellular matrix (ECM) formation, providing support and enabling new tissue formation. It can be noticed that the best results were obtained for pure PLA nanofibers. As revealed in Figure 5, a significant decrease in density was observed with the addition of metal nanoparticles, which can be explained by the high molecular weight of introduced NPs. This negative effect was slightly neutralized by the presence of polymeric coatings. Interestingly, a similar phenomenon was observed during the porosity study only for part of the tested sample. Figure 5 shows that the addition of Au NPs (both bare and PP/PANI coated) resulted in the porosity increase. On the other hand, the addition of Ag or Pt NPs caused a porosity decrease. However, the biggest change was spotted for PP coatings, which can be related to their hydrophobic nature. Noteworthy, prepared nanofibrous 2D mattes were relatively thick to act as a scaffold for nerve tissue regeneration. Porosity

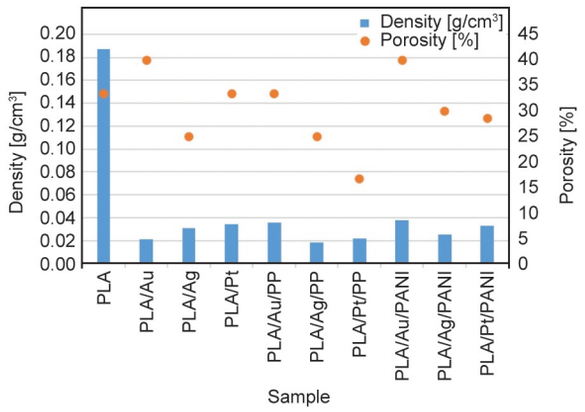

Figure 5. Density and porosity of the sample.

values can be assigned to the nanofibers' distribution and diameter as well as the stationary mode (no needle movement/sliding) of electrospinning as well as the solid nature of the collector and its relatively small diameter. Moreover, it is possible that the presence of conductive nanoparticles had an impact on the fibrous mesh formation process due to extra interactions repelling during electrospinning, which occurs in the electric field, resulting in more distant fibers embedding on the collector [28–30].

The ability of aqueous solutions sorption is an important parameter that determines the ability of the biomaterial to act as a scaffold during tissue regeneration and provide appropriate environmental conditions for cell proliferation. As shown in Figure 6, newly developed nerve guide conduits were capable of swelling with both distilled water and simulated body fluid, which makes it possible to accumulate small amounts of bioactive compounds such as growth factors or some other biochemical cues [30]. It can be observed that swelling abilities are significantly lower than NGCs in the form of hydrogels prepared from natural polymers such as collagen,

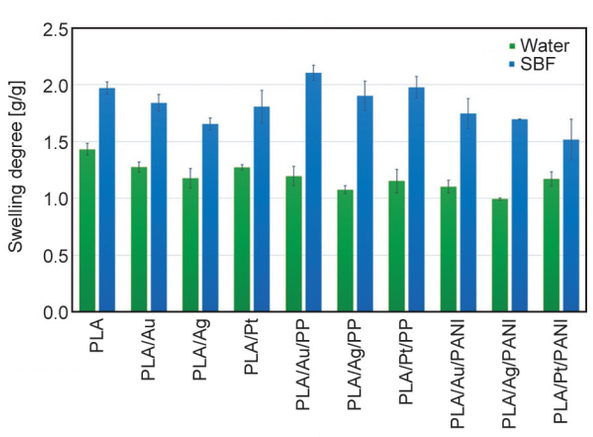

Figure 6. Swelling properties of the prepared samples.

gelatin, or chitosan. Therefore, it can be assumed that they will be more stable under in vivo conditions and will maintain their integrity as well as architecture. No clear relationship between chemical composition and swelling degree can be spotted, which varies around 1 g of swelling medium per 1 g of the biomaterials. Noteworthy, all materials were more prone to absorb SBF, which is rich in various inorganic salts, than pure water.

Gold, silver, and platinum nanoparticles are known for their conductive properties. The study showed a significant increase in conductivity for nanoparticles coated with conductive polymers as shown in Figure 7. The addition of polypyrrole increased the conductivity of gold nanoparticles by 60%, silver nanoparticles by 24%, and platinum nanoparticles by 34%. The addition of polyaniline increased the conductivity of gold nanoparticles by 13%, silver nanoparticles by 10%, and platinum nanoparticles by 14%. Such results clearly show that the concept of NPs additional coating results in the electrical properties enhancement and is superior to traditional polymeric shells [16–24].

Obtained nanoparticles were further used to prepare fibrous biomaterials with potential conductive properties. As shown in Figure 7, the addition of the metallic NPs to the PLA solution resulted in the formation of the nanofibers with increased conductivity compared to pure PLA. Importantly, NPs coating with PP or PANI resulted in the further increase of this parameter. The highest conductivity was measured for PLA/Ag/PP and PLA/Ag/PANI samples. Such results show that prepared biomaterials can be used for electrostimulation of nerve cell growth and proliferation and may be used as potential NGCs.

One of the important features of biomaterials is their durability. Nerve guide conduits must preserve structural integrity during preparation for implantation, seeding with cells and under in vivo conditions. Figure 8 presents the results of tensile strength analysis carried out on newly developed membranes. Pure PLA membranes exhibit quite satisfactory mechanical durability, which is almost 0.9 MPa. However, our study has shown that the addition of metallic nanoparticles positively affected this property in all cases. Nevertheless, the increase is mediocre and does not significantly change the durability of the biomaterials. It can be noticed that the highest TS was obtained for membranes modified with silver nanoparticles. The presence of polymeric coating (PP/PANI) also had a positive impact on tensile strength; however, it can be considered as very small or negligible. This phenomenon can be explained by better mixing polymer with PLA matrix than raw metallic NPs.

NGCs are tubular structures with the aim to restore nerve function after damage and can come as non-permeable, semi-permeable and fully permeable biomaterials. They act as a bridge that helps to link to served nerves. They play multiple roles, including protection and guidance. Prevention of scar tissue, together with providing an appropriate microenvironment for axon regeneration, are the most important features of NGCs [28–30]. As shown in Figure 9, prepared nanocomposites were characterized by nanofibrous architecture. Most of the fibers have random orientation, and only a small number of them exhibit slight alignment. It can be observed that fibers are of nanometric, homogenous diameter for PLA/Ag/PP and PLA/Pt/PP, while PLA/Au/PP biomaterial

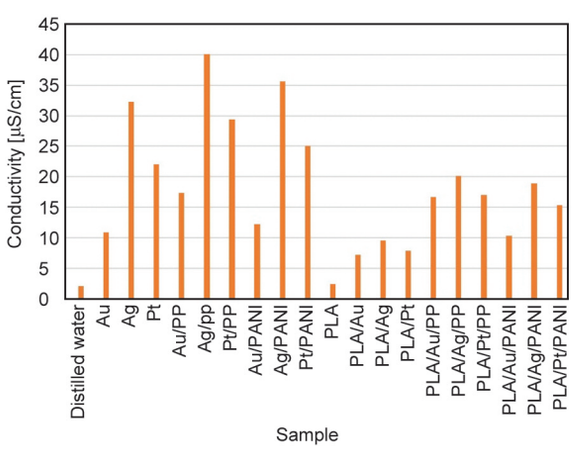

Figure 7. Conductivity of the prepared nanoparticles and ready membranes.

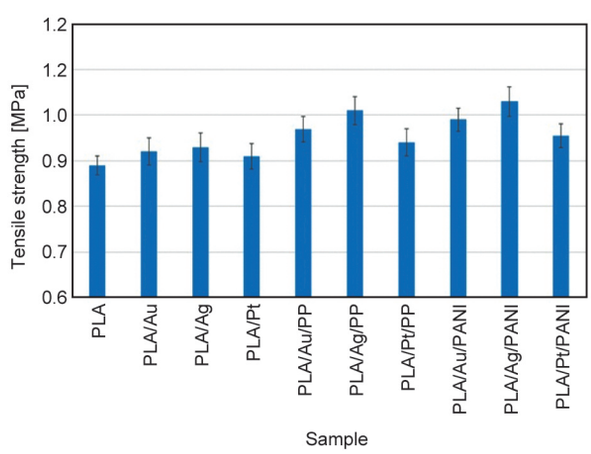

Figure 8. Mechanical properties of nanofibrous materials.

contains fibers with high thickness dispersion. Interestingly, depending on NPs type, different average pore diameters can be observed from 15 µm for PLA/ Ag/PP to 5 µm for PLA/Pt/PP, which clearly indicates that conductive nanoparticles in the electric field during electrospinning affect the fibers formation process.

Figure 10 presents the results of the roughness analysis. It can be observed that all three samples are characterized by homogeneous, rough structures, which

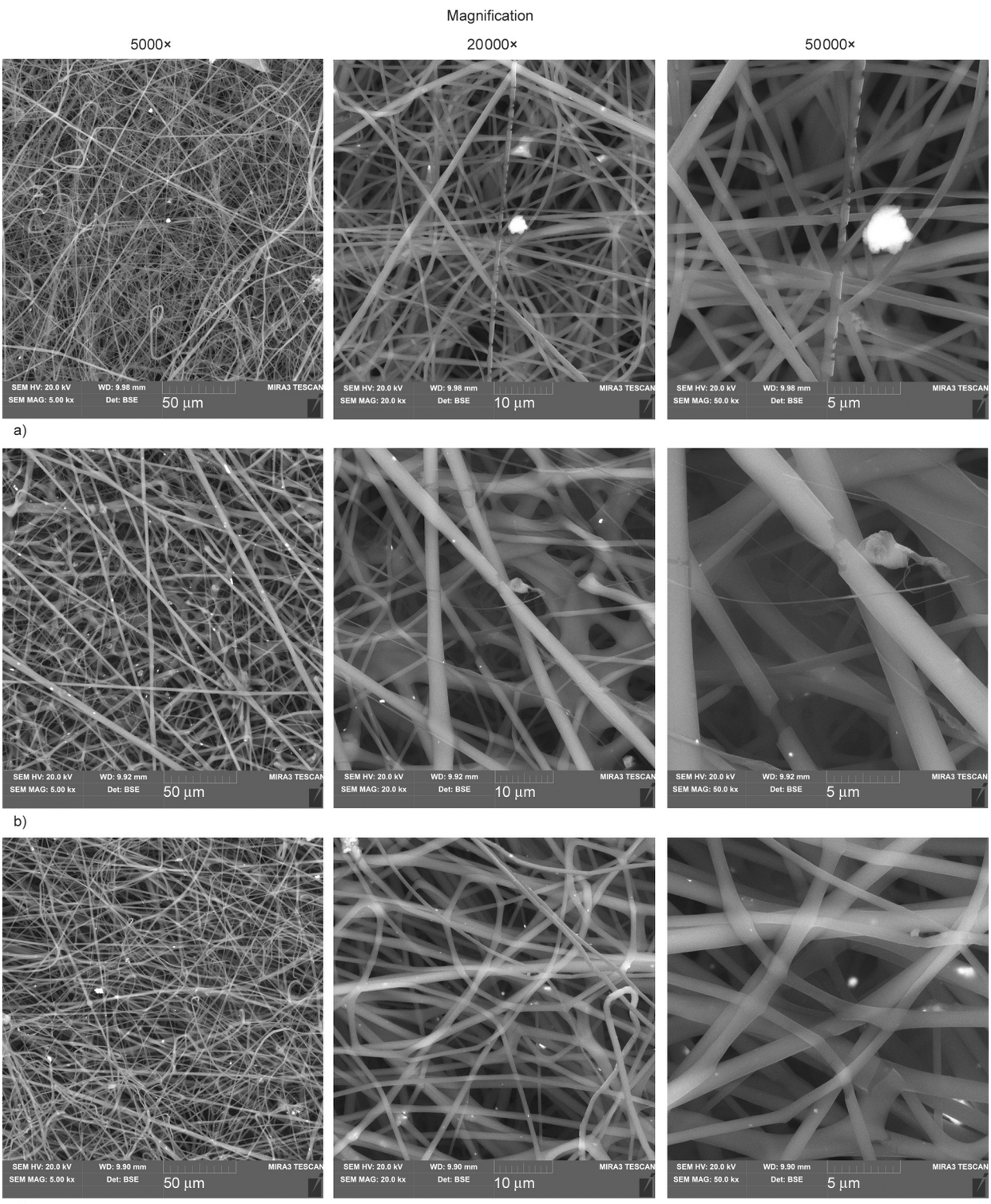

Figure 9. SEM microphotographs of the a) LA/Ag/PP nanocomposites, b) PLA/Au/PP nanocomposites, and c) PLA/Pt/PP nanocomposites.

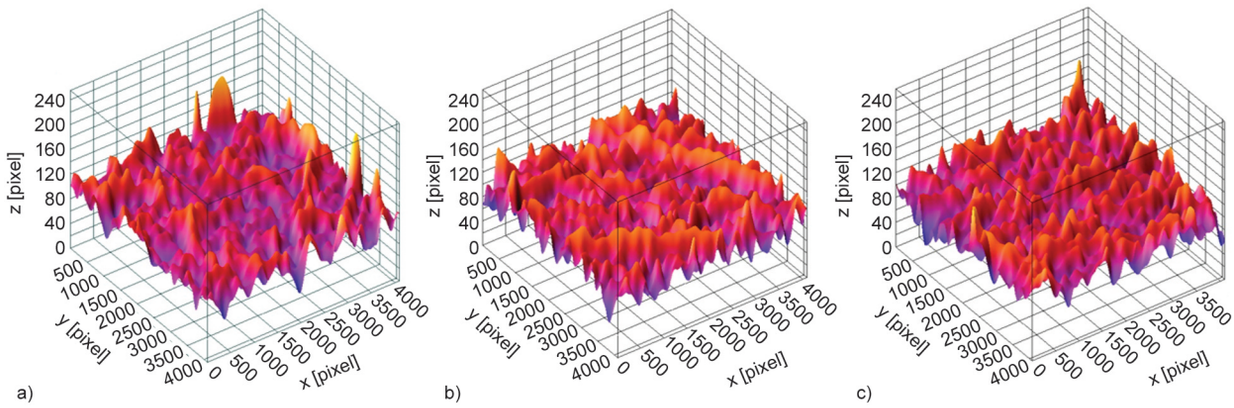

Figure 10. Surface morphology: a) PLA/Ag/PP nanocomposite; b) PLA/Au/PP nanocomposite; c) PLA/Pt/PP nanocomposite.

is a consequence of nanofibers' random alignment. Such morphology provides appropriate conditions for cell attachment and proliferation. Noteworthy, the most uniform surface was obtained in the case PLA/Au/PP nanocomposite. Pores size has been determined using software dedicated to SEM microscope. Their average size ranged mostly between 5 to 10 μm; however, due to their random nature, their diameter is heterogeneous. Nevertheless, it provides an appropriate architecture to enable growth factors, nutrients and oxygen delivery as well as removal of CO_2 and metabolites.

NGCs of the latest generation should not only provide protection of the newly formed, immature nerve tissue but also accelerate the regeneration process, which can be achieved by chemical, electrical or mechanical stimulation [28–30]. Figure 11 shows SEM microphotographs of biomaterials together with elemental mapping. As it can be noticed, depending on the nanoparticles type, their placement is at different depths which is correlated with their affinity to PLA nanofibers. As shown in Figure 11, silver NPs. are homogeneously spread on superficial fibers. However, their polypyrrole coating is quite thin.

SEM analysis revealed that all PLA matrix modifications with polymer-coated NPs were not the same depending on the nanoparticles. As shown in Figure 11, Ag NPs were embedded most successfully, and their presence on the internal fibers of the material was confirmed. An XRD analysis was carried out to investigate the presence of metallic nanoparticles in the structure of biomaterials further. As shown in Figure 12, for all investigated samples, reflexes typical for nanoparticles were spotted, which confirms the presence of all types of NPs in all 3 cases since it provides better insight into materials' chemical composition due to better X-ray penetration during analysis compared to EDS. Such results prove that the use of natural extracts rich in reductive compounds such as aldehydes. They provide the possibility to obtain metallic nanoparticles stabilized with non-toxic substances with favorable

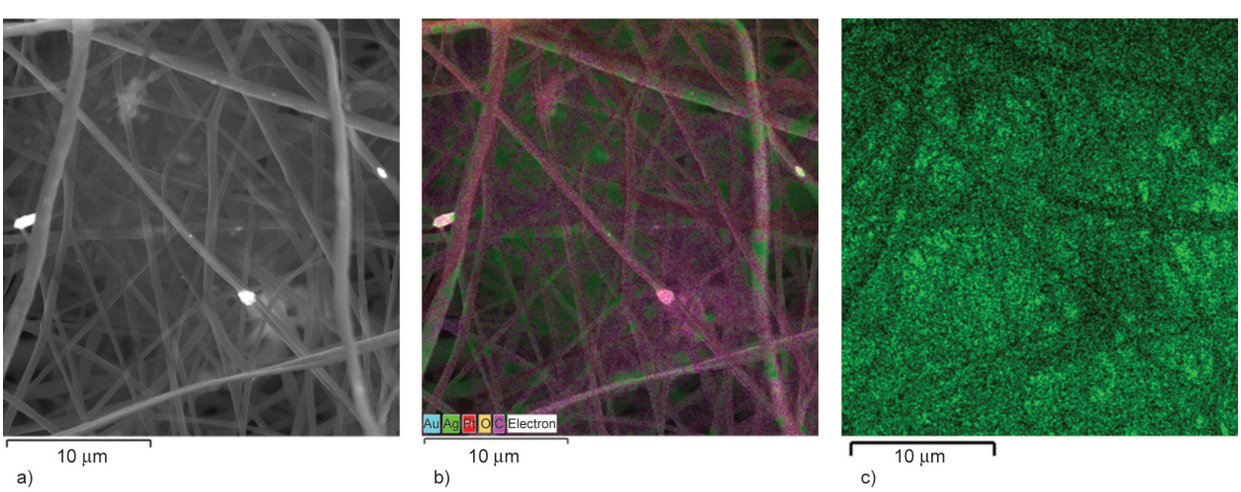

Figure 11. SEM microphotographs of the nanocomposites with elements mapping: a) PLA/Ag/PP magnification 50 000×, b) elemental mapping, c) Ag mapping.

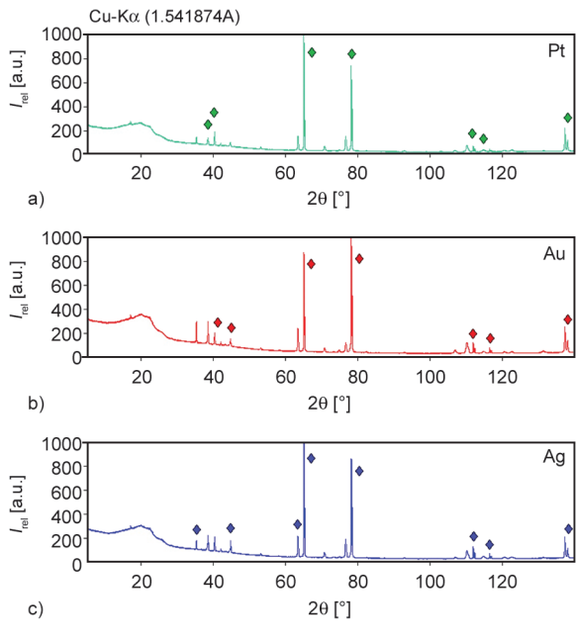

Figure 12. XRD analysis of: a) PLA/Pt/PP nanocomposites; b) PLA/Au/PP nanocomposites; c) PLA/Ag/PP nanocomposites.

antioxidant properties. Thus, they positively affect the biological outcome of the ready biomaterials due to the possibility of reactive oxygen species neutralization.

3.4. Determination of the cytotoxicity of the obtained nanocomposites

To test the biocompatibility of the samples, a direct cytotoxicity test was performed to verify cell morphology, detachment from the bottom of the cell culture flask, their vacuolation or violation of cell membrane integrity. The results are shown in Figure 13. After 72 h of cell culture, no negative interactions between the cells and the material were observed. After this time, the mouse dermal fibroblasts formed a uniform layer and no reduced growth was observed compared to the control. Few round cells and no intracytoplasmic granules were observed. The cells are tightly connected to each other, and there are no free

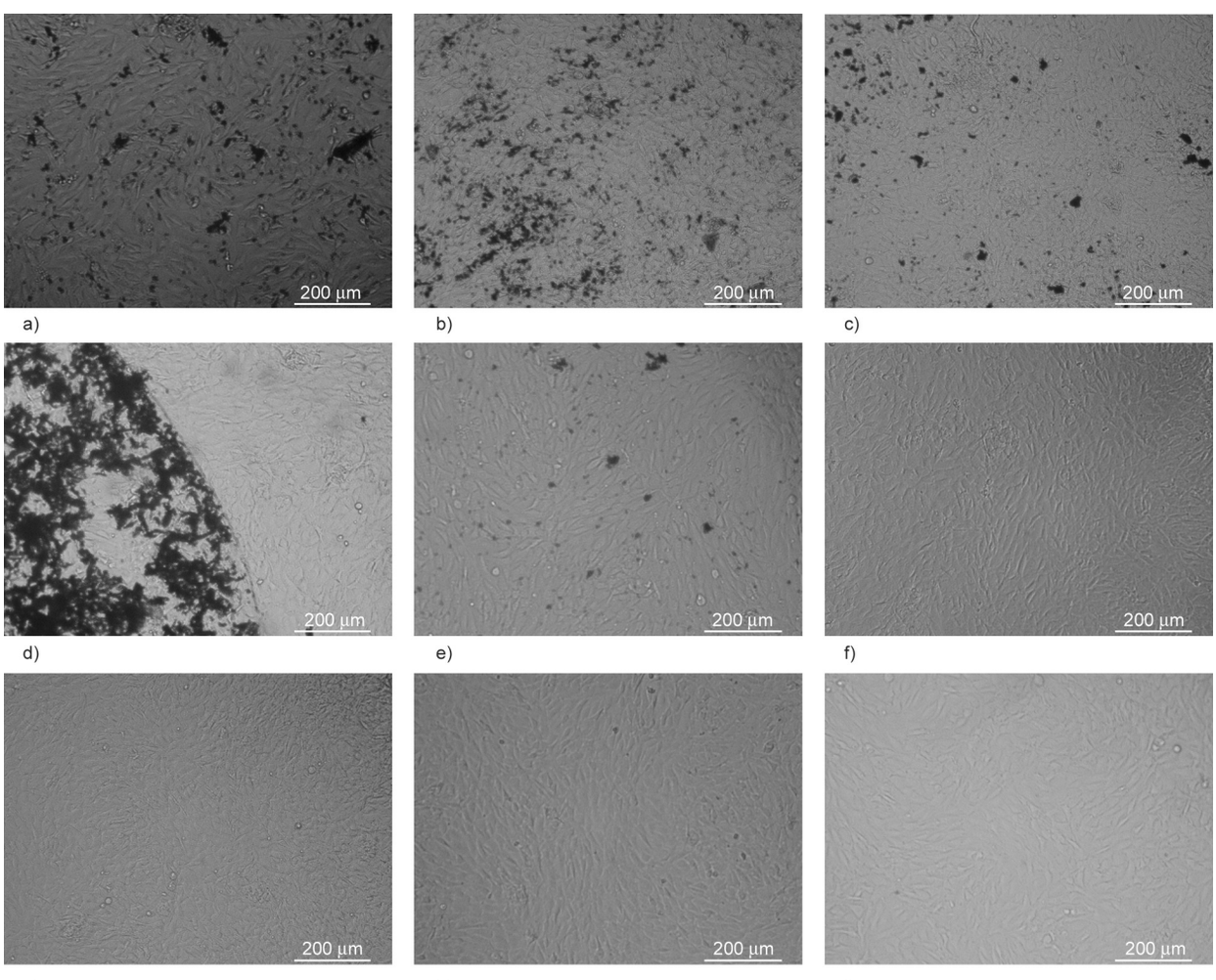

Figure 13. Qualitative cytotoxicity assessment of the developed samples on mouse fibroblasts after 72 h of cell culture (40× magnification): a) Au/PP; b) Ag/PP; c) Pt/PP; d) u/PANI; e) Ag/PANI; f) Pt/PANI; g) PLA; f) PLA/Au/PP; i) PLA/Pt/PANI.

spaces between them. The fibroblasts have a normal, spindle-shaped morphology, and no abnormalities can be observed. The morphological qualitative evaluation showed that the biomaterials have no cytotoxicity according to the ISO 10993-5 standard for biomaterials. Overall, the study confirmed the biosafety of the developed nanomaterials and nanocomposites. No signs of material degradation have been spotted, which is typical for PLA-based biomaterials which hydrolysis, depending on the average molecular mass of the raw polymers, starts approximately 3 months.

The most promising biomaterials were further investigated over their potential as nerve guide conduits. Therefore, direct cytotoxicity studies were carried out on glial cells. As shown in Figure 14, after 72 h, a confluent monolayer of cells was obtained in each case. 1321N1 cells had elongated, normal morphology. No detached cells or apoptotic morphological changes can be spotted. Also, no cell shrinkage is visible nor vacuoles in the cytoplasm, which confirms the lack of cytotoxic effect of the evaluated biomaterials on the astrocytoma cells [31–34].

4. Conclusions

Nerve guide conduits constitute a promising alternative to traditional autografts. This method of treatment is associated with lower patient discomfort and provides better healing results. In this work, a novel type of biomaterials was obtained using electrospinning process according to Green Chemistry and 6R rules. To increase functionality of standard PLA NGCs, products were modified with three types of metallic nanoparticles coated with conductive polymers (PP, PANI) to preserve their conductivity and better integrity with polymeric matrix. NPs were prepared using natural extracts which confirmed TEM analysis. Biomaterials were investigated over their chemical structure, morphology, porosity and conductivity. Finally, potential NGCs were verified over their cytotoxicity to L929 mouse fibroblasts and 1321N1 human glial cells. The obtained results clearly demonstrated that prepared nerve guide conduits have great potential in the field of nerve tissue regeneration and nerve defect treatment. Our future study will focus on the long-term effect of nanoparticles on potential cytotoxicity, cell proliferation, morphology and cell cycle, as well as the possibility of three-dimensional new tissue formation. Additionally, electrostimulation tests using platinum wires will be proceeded to verify whether PLA-based NGCs have accurate conductivity to provide a positive effect on cellular behaviors and stimulate the regeneration process, resulting in the obtainment of a tissue with full biological and mechanical functionality and maturity. Also, mechanical integrity and durability

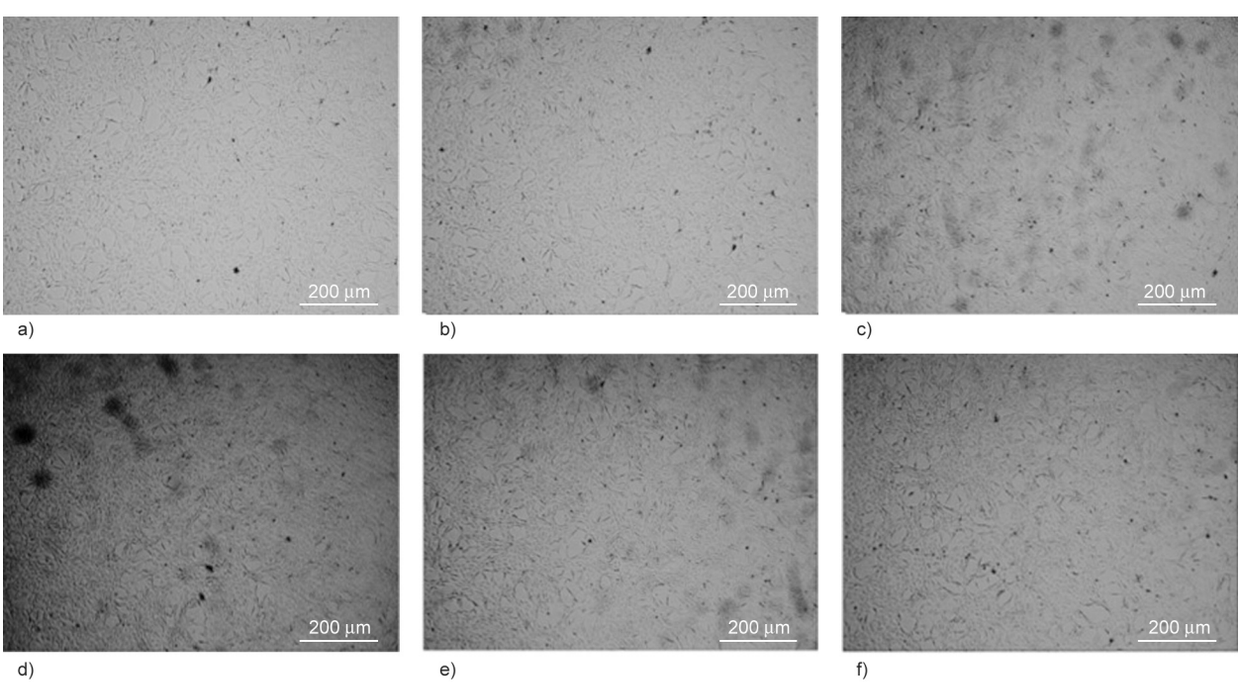

Figure 14. Qualitative cytotoxicity assessment of the developed samples on 1321N1 human glial cells after 72 h of cell culture (40× magnification): a) Au/PP; b) Pt/PP; c) Au/PANI; d) Pt/PANI; e) PLA/Au/PP; f) PLA/Pt/PANI.

in time will be investigated to determine possibility of full tissue replacement after favorable period of regeneration.

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