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Nerve Guidance Channels Based on PLLA-PTMC Biomaterial

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ABSTRACT: Biodegradable polymers of poly(lactic acid) (PLLA) and synthesized in-house poly(trimethylene carbonate) (PTMC) with admixture of water-soluble methyl cellulose (MC) were used for development of nerve guidance tubes for peripheral nervous system regeneration after injury. Fabrication method involved phase separation of viscous dixane solution of polymers mixture seat on a rod in a proper nonsolvent, which resulted in tubular structure of large porosity. Influence of electron beam sterilization on molecular weight, thermal properties of the polymers, and mechanical performance of the tubes was evaluated. Admixture of hydrophilic MC to synthetic polymers resulted in modification of mechanical properties of the channels. Extraction of MC showed potential of the tubes for releasing water-soluble bioactive molecules, such as for instance growth factors. Basic *in vitro* MTT and LDH assays showed no cytotoxic effect of manufactured tubes, therefore, animal experimentations may be considered. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: biomaterials; electron beam irradiation; nerve regeneration; poly(L-lactic acid); poly(trimethylene carbonate)

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INTRODUCTION

Peripheral nervous system is composed of neurons and glial, comprissing Schwann cells, or satellite cells. The function of neurons is to carry signals between the brain and the rest of the body, whereas Schwann cells support the neurons, enhance the speed of the electrical signals and produce proteins for neuronal growth. Peripheral nerve lesions are serious injuries affecting 2.8% of trauma patients annually, and generally lead to lifelong disability.[1] The worst case of a peripheral nerve trauma is the total disruption of the nerve, which requires realignment. Nerve stumps tend to retract and their primary suturing is possible only when no tension is generated. If the gap between nerve stumps is too large, an appropriate harvested nerve or a device for bridging and guiding the axons has to be used. In the case of peripheral nerve regeneration, medical problem typically involves the loss of innervation in arms or legs, leading to the loss of motor and sensory function. The strategies developed for nerve repair can be roughly classified into the following categories[2]: (1) end-to-end suturing of the nerve stumps – this is practically possible only in the case when a nerve bundle is cut without substancial loss, (2) transplantation that involves nerve autografting – at present it is the most sucessful technique. This method has been shown to be very effective as it avoids tension across the repair site, unfortunatelly affects donnor site.[3] Allografts and xenografts have also been used for peripheral nerve regeneration, despite the possibility of an undesirable immune response, and (3) a bridging technique, which involves implantation of biocompatible, and often bioactive tube. The bridging channel will secure a space for an injured nerve regrowth, guide developing axons into proper direction and may stimulate axons propagation.[4] Additionaly, the technique avoids tension and neuroma formation occuring quite frequently in the case of end-to-end connections and nerve transplantation.[5]

A wide variety of materials have been suggested for the production of artificial devices for nerve repair, including biocompatible nondegradable and degradable materials,[6,7] such as for instance silicone,[8] polyglycol,[9] poly(ε-caprolactone) (PCL),[10] polylactides, and their copolymers.[11] There have been also efforts to apply tubes with inner three-dimensional scaffold of for instance biodegradable hydrogel, that would support axonal growth. Nevertheless, a constant disagreement that remains unsolved is whether utilization of a filling is beneficial for the improvement of nerve reconstruction.[12] Among biodegradable polymers, polylactides are widely used in implant devices. Poly(lactic acid) (PLLA) has an established position as commercial biomaterial with its utilization primarily to load bearing applications due to relatively high strength and modulus.[13,14] There were several partially sucessful trials with PLLA-based

flexible porous or membrane-like materials, as well as another rigid biodegradable polymer of PCL for nerve regeneration purposes.[15,16] Neverthless, introduction of soft, flexible segments, or co-components, by copolymerization or blending route, become advantageous for utilization in devices where high elasticity is essential. Moreover, biodegradation kinetics may be tailored to the need of particular application by choosing constituents of various degradation profile and altering their composition. Amorphous, highly flexible, poly(trimethylene carbonate) (PTMC) seems to be an interesting candidate to introduce modifications to rigid PLLA.[17–20] This linear polymer of poly(alkylene carbonate)s family, with its glass transition temperature (T_g) of about −15 to −20°C is rubberry material at room temperature. Nevertheless, at the temperature of human body, about 37°C PTMC becomes too soft and tacky for application as biomaterial. Only PTMC with signifficantly high molecular weight possesses good elasticity and strength. Long synthesis time at relatively harsh environment results in PTMC with high molecular weight, otherwise common synthesis methods yield polymer with poor mechanical properties.

To effectively increase the molecular weight and therefore to modify the properties of PTMC, ionizing radiation by means of electron beam (EB) can be applied. Linear chains of PTMC undergo radiation-induced cross-linking, which in turn may result in improved mechanical properties of this polycarbonate.[21] PTMC has been investigated for biomedical applications in soft tisse engineering,[22,23] drug delivery systems[24] and scaffolds for nerve regeneration.[25] Alternatively, natural polymers are characterized by superiour biocompatibility, since they may mimic natural tissue behavior, and when composed with synthetic polymers can influence mechanical properties and biodegradation profile of the biomaterial or a device.[26] Polysaccharides that were utilized in nerve regeneration applications include collagen,[27] chitin,[28] alginate,[29] and other natural polymers or their derivatives. It is also feasible to apply natural polymer to introduce or modify porosity, and therefore, elasicity of the synthetic polymer matrix since for instance water soluble polysaccharide can be easily washed away through extraction process.

The aim of the present investigations was to design a porous guidance tubes by deveopment of biocompatible materials through blending route for the production of nerve guides of sufficient mechanical properties that can be easily handled and sutured in. The candidate materials were PLLA/PTMC blend with and without addition of methylcellulose (MC), a water-soluble polymer of natural origin with potential for biomedical industry.[30] A strightforward method of manufacturing of porous conduits was elaborated and obtained products were characterized through mechanical testing, evaluation of morphology, molecular weight, and thermal properties of comprising polymers in order to measure changes induced by chosen sterilisation method of EB irradiation. Biocompatibility, a critical factor of any medical device, was primarily assesed through cytotoxicity testing by two quantitative tests of LDH and MTT with respect to mouse fibroblast cells.

EXPERIMENTAL SECTION

Materials

Poly(L-lactic acid), PLLA of viscosity-average molecular weight, M_v of 62 kg mol^{-1} was purchased from Dow-Cargill. Its gel permeation chromatography (GPC)-determined number-average (M_n) and weight-average (M_w) molecular weights are shown in Table I. PLLA was purified by dissolving in chloroform (10%, wt/vol) and precipitation into 10-fold volume of methyl alcohol. PTMC was synthesized in-house and the procedure is described below. Methyl cellulose (MC) of medium viscosity (400 cP in 2% aqueous solution at 20°C) was purchased from Sigma-Aldrich. It was purified by precipitation onto acetone from its water solution. Solvents and other chemical compounds of analytical grade, unless stated below, were purchased form Sigma-Aldrich and used without further purification.

Table I. Number-Average (M_n), Weight-Average (M_w) Molecular Weights [kg mol^{-1}] and PD (M_w/M_n) of PTMC, PLLA, and Polymer Blend PLLA/PTMC Taken from Manufactured Tubes, Nonsterile and Sterilized with EB of 25 kGy

	Nonsterile			EB of 25 kGy		
Polymer	M_n	M_w	PD	M_n	M_w	PD
PLLA	79	155	1.9	41	120	2.9
PTMC	63	94	1.5	54	99	1.8
PLLA/PTMC	56	122	2.17	42	103	2.43

PTMC Synthesis

PTMC is not available commercially therefore the polymer was synthesized in our laboratory. The method for PTMC polymerization was based on that described in the literature for similar compounds such as polylactides and PCL[31] and tailored for polymerization of the polycarbonate. Trimethylene carbonate monomer (Boehringer Ingelheim, Germany) was purified before polymerization through resublimation. Synthesis was performed in an anhydrous tetrahydrofuran with diethylaluminum ethoxide (Aldrich) initiator. Diethylaluminum ethoxide was added to monomer in the 1000 monomer to initiator molar ratio and the polymerization was performed for about 15 h at room temperature under argon atmosphere. Afterwards the polymer was dissolved in chloroform and purified by precipitation onto methyl alcohol. Polymer washed with methanol was dried under reduced pressure at room temperature to a constant weight.

Molecular structure of obtained PTMC was confirmed by proton nuclear magnetic resonance (^{1}H-NMR) technique. The spectrum was recorded at 300 MHz on Varian Innova spectrometer, using $CDCl_3$ as a solvent (Merck, Germany) – the data are not shown here. Molecular weight was determined by a GPC, as described below, and is presented in Table I.

Thermal Characterization

Transition temperatures of the PLLA, PTMC, and the blends were determined by differential scanning calorimetry (Q200 MDSC, TA Instruments). Samples of 5–6 mg sealed in aluminium pans were analyzed in the temperature range −50–200°C at a heating/cooling rate of 10°C min^{-1} under an inert gas atmosphere. The data were collected through three scans: first heating, cooling and second heating, and each of the characteristic transitions temperatures was estimated based on the scan that show clear transitions as denoted in the text with presented data. The T_g was taken as the midpoint of the heat capacity change; the

melting temperature was determined in a top of the melting peek. Degree of crystallinity (DC) was calculated based on area under endothermic transition curve with assumption that only polylactide segments form crystalline phase and the heat of melting (ΔH) of 100% crystalline sample is equal to 93.64 J g^{-1}.[32] Crystallinity degree was recalculated for the actual amount of the PLLA in sample (50% in the case of PLLA/PTMC blend).

Molecular Weight Determination

Gel permeation chromatography (GPC) measurements were done to determine M_n and M_w molecular weights, and molecular weight distributions (M_w/M_n) of polymers. System equipped with a P580 pump (Dionex), two columns of 10 and 5-μm pore size (Knauer) and three detectors: Viscotec Ralls Detector (static light scattering at angle of 90° at a wavelength of 670 nm) and Viscotec Dual Detector 250 (refractometer/viscometer) was used.

Dichloromethane was used as an eluent at 30°C at a flow rate of 0.8 mL min^{-1}. Polystyrene standards of narrow molecular weights were used to calibrate retention times for the detectors. Sample concentrations in the range 2–10 (wt/vol %) and injection volumes of 100 μL were applied. Solutions were filtered prior to injection into the GPC system through 0.45 μm PTFE membrane filters (Sartorius).

Tube Manufacturing

Method of tubes fabrication developed in our laboratory is schematically presented in Figure 1. Number of compositions were tried and, at the base of further physicochemical evaluation, currently exploited tube composition and fabrication method is briefly described as follows. Polymers of PLLA, PTMC in 1 : 1 wt. ratio were dissolved in dioxane at total concentration of 20 wt/vol %, which resulted in very viscous liquid. Additionally MC was dispersed and eventually dissolved in the solution at 1 : 10 wt/wt % ratio with respect to total synthetic polymers mass. Standard injection needles of 1.1-mm diameter, acting as a mandrel were repeatedly dipped in the polymer solution and a nonsolvent with respect to synthetic polymers, which was water or aqueous solution of alcohols (methanol, ethanol, isopropyl alcohol). Eventually, isopropanol and water in 3 : 7 vol/vol % proportion was used. Polymer recovery from the solution was triggered by phase separation in the nonsolvent bath resulting in tubular porous structures of about 3 cm length developed on a mandrel. Polymer tubes were removed from needles, washed in water and alcohol for 24 h, dried and separately packed.

Sterilization by ionizing radiation with a standard sterilization dose of 25 kGy was applied. Tubes were irradiated by an EB from the ELU-6 linear accelerator of 6-MeV energy. Actual dose and the dose rate were monitored by alanine dosimeter. The average dose rate was 5.8 kGy min^{-1}.

To evaluate impact of MC on the properties of the tubes extraction in phosphate buffer saline (PBS) solution was conducted and followed by tensile properties and morphology examination. Sets of tubes with and without MC, both unirradiated and sterilized were immersed in the solution for 10 days at 37°C,

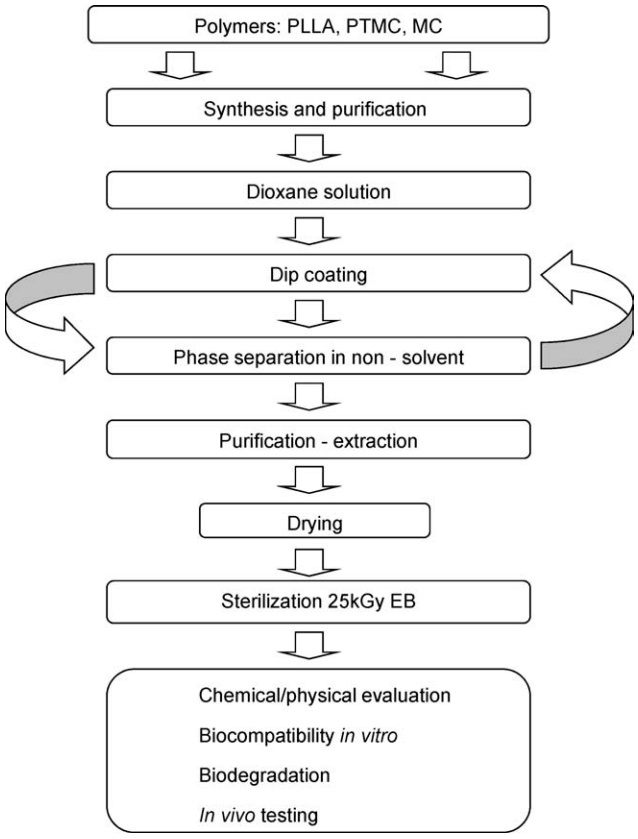

Figure 1. Schematic diagram of nerve guidance tubes manufacturing procedure. Current article covers investigations on the manufacturing process, physical–chemical testing and fundamental cytotoxicity testing as a primary biocompatibility assesment.

then removed, rinsed with water and dried. It is expected that most of the MC incorporated in the polymer blend was removed during 10 days of extraction, see Discussion later.

Tube Morphology - SEM

To examine a cross-section of manufactured tubes they were frozen in liquid nitrogen and transversally broke. Scanning electron microscopy pictures of gold-coated cross-sections of the tubes were taken with Hitachi TM-1000 machine.

Tensile Properties Evaluation

Mechanical properties of tubes were investigated by tensile testing machine (Z2, Zwick Roell, Germany). Samples were fixed in dedicated cylindrical clamps. An internal supporting cylinder was applied to prevent deformation of the tubes in the clamps. The speed of the crosshead was 3 mm min^{-1} with an upper force limit of 100 N. Experiments were run at room temperature. The specimen deformation was derived from the grip-to-grip separation, which was initially set at 10 mm. A mean value of five different measurements was determined and a standard deviation was calculated.

Cytotoxicity Testing

Two commercial tests were applied: TACS™ MTT Cell Proliferation Assay (Trevigen) and LDH Cytotoxicity Detection Kit (TaKaRa) for cytotoxicity evaluation. Samples for tests were

prepared according to the ISO standard 10993-12 "Biological evaluation of medical devices – Sample preparation and reference material," and the tests were conducted in accordance with ISO 10993-5 Part 5: "Tests for in vitro cytotoxicity." Indirect method of extracts testing was applied. The surface area was used to determine required volume of extract; 1 mL of solvent was used for every 3 cm^2 of surface area. Culture medium (Dulbecco's modified Eagle Medium) without serum was used as a solvent for elution. Extraction was performed at 37°C for 24 h in water bath with shaking.

Cell line used in cytotoxicity evaluation was the L-929 mouse fibroblast (ATCC European Collection of Cell Cultures), which are used most extensively for testing biomaterials and medical devices mainly because they are easy to maintain in culture and produce results that have high correlation with specific animal bioassays. Cells at density of 1×10^5 cells/mL were cultured in flat-bottomed 96-well tissue culture plate and incubated at 37°C in 5% CO_2 for 24 h. In parallel with tested samples different controls were run in the assay – cells with only medium, with 1% Triton X-100 and with commercially available polyethylene.

For LDH tests, the incubation medium was replaced by 100 μL of fresh medium, and 100 μL of test eluats or controls were transferred into corresponding wells. Plates were incubated at 37°C in 5% CO_2 for 24 h. After the incubation microtiter plate was centrifuged for 10 min and then 100 μL of supernatant from each well was transferred into corresponding wells on 96-well flat bottom plate. Subsequently, 100 μL of the reaction mixture was added to each well and incubated in the dark for 30 min at room temperature. The absorption of samples was measured at 490 nm with ELISA microplate reader.

To perform MTT assay the medium was removed after the incubation and 100-μL extract of test products per well were added. The plates were incubated at 37°C in 5% CO_2 for 48 h. After that, 10 μL of MTT Reagent was added to each well and plates were incubated for 2 h at 37°C. When purple precipitate was clearly visible, 100 μL of Detergent Reagent was added to each well. Plates were left covered in the dark for 4 h. The absorbance values were recorded at 570 nm.

RESULTS AND DISCUSSION

Choosing the most favorable polymer composition for a particular application should be preceded by reviewing the requirements of the product. Sufficient mechanical strength combined with high elasticity of the guidance tubes that will assist regeneration of broken peripheral nerves is crucial. To produce a tube designed for our needs, it was assumed that toughness can be provided by a durable polymer of PLLA and flexibility can be tailored by admixture of rubber-like polymer such as PTMC as well as introducing porosity by applied manufacturing technique and addition of a porogen that may be extracted.

PTMC that is unavailable commercially, yet it is known as a biomaterial with high potential in medical field, was polymerized in house. The synthesis has yielded a highly flexible but not tacky polymer of relatively low M_n molecular weight of about 63 kg mol^{-1}. Molecular structure of PTMC was confirmed by proton NMR (data not shown). Thermal characterization by DSC revealed amorphous material with its T_g of about −18°C, that is common for PTMC.[33] Basic characteristics of synthesized polymer is presented in Tables I and II.

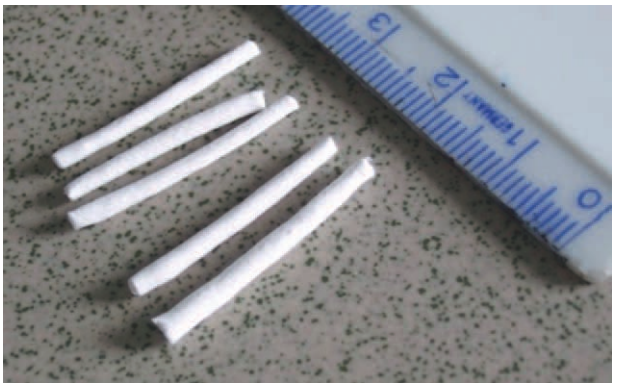

Figure 2. Photograph of manufactured nerve guidance channels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Common solvent for synthetic polymers of PTMC, PLLA, and MC that was used in further experiments was selected based on the solubility data[33] and experimental work with these polymers. Relatively safe dioxane was chosen for solutions preparation as the one that dissolves the three polymers. As the procedure of tube production includes recovery of polymers from their solutions, several compositions of the nonsolvent, in which the polymers precipitate were considered. Those included water, methyl alcohol, ethyl alcohol, isopropyl alcohol, and water solutions of these alcohols in various concentrations. Resulting tubes had diverse mass, mechanical strength, wall thickness, and porosity. Pores were created in the process of phase separation, therefore, their size and uniformity strongly depends on the polymer concentration and a nonsolvent composition. Isopropyl alcohol : water mixture of 0.3 : 0.7 (v/v) nonsolvent was eventually selected. Dipping of the mandrel in the polymers solution and the nonsolvent was repeated 10 times to achieve sufficient thickness of the tubes. A photograph of manufactured nerve guidance channels after extensive rinsing and drying is presented in Figure 2.

SEM picture of the cross-section of tubes made of PLLA/PTMC blend is shown in Figure 3(a,b). The tube wall is of about 0.6-mm thick. Pores are relatively large, of up to 100 μm and not uniform. Moreover, there is no explicit difference in the morphology of the tube if MC was added to the blend [Figure 3(c)] besides that the tube walls are thicker. Porosity evidently affects mechanical properties of the final product, as compared to nonporous material (see below), but depending on the application, its presence can be acceptable if is related to another beneficial feature of the product. In this particular application, porosity may allow for easier fluids and gas exchange between regeneration environment of inner tube space and surrounding, for example, connective tissue, avoiding the build up of pressure due to fluid retention. Encrustation of outside tissue on the surface and into the porous structure of the tube walls is another factor that increases implant–host association and may lead to

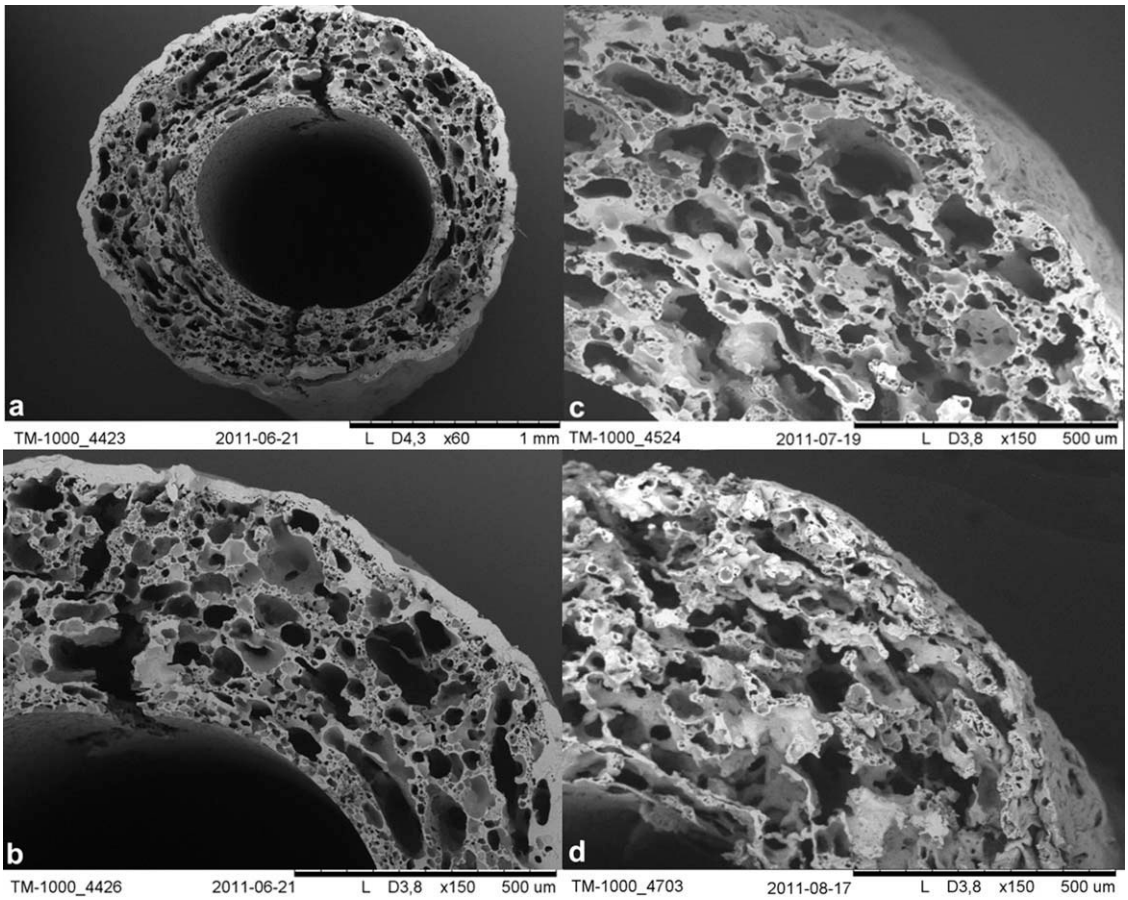

Figure 3. SEM photographs of cross-sections of manufactured nerve guidance tubes. (a) and (b) PLLA/PTMC, (c) PLLA/PTMC with 9 wt % MC addition, (d) as in (c) after 10 days extraction in PBS.

accelerated biodegradation of the tube, while maintaining a barrier to fibroblasts and other cells.[6] Therefore, we assume that presence of porous morphology is beneficial for utilization of the tubes as nerve guidance channels.

Every medical device supposed to contact patient body has to be sterile. Common sterilization techniques applied to either large quantities of disposable products or single reused devices must be validated for certain product to assure sufficient efficiency in killing microorganisms.[34] Biodegradable polymeric products must not be sterilized by dry or moisture heat methods due to possible melting or softening of the polymer material resulting in de-shaping of the product and induction of potential degradation of polymer through hydrolysis. In this case, glass transitions temperatures of investigated polymers are low, moreover ester bond in PLLA main chain easily undergoes hydrolysis while in contact with water.[35] In such case, low temperature method of ethylene oxide (EtO) is frequently used, for instance for PLLA fixation devices.[36] This sterilizing agent must diffuse into all voids of the product and afterwards diffuse out, therefore, this method is not recommended for porous materials. Similar disadvantage related to active gas penetration can be encountered for plasma sterilization technique. In this conditions, sterilization with ionizing radiation seems to be a good alternative for porous scaffold comprising biodegradable polymers of low T_g. Determination of the influence of applied EB sterilization was done by comparison of polymer characteristics and tubes properties before and after application of 25 kGy dose from electron accelerator.

It is known that PLLA degrades readily under influence of ionizing radiation, which consequently causes deterioration of mechanical performance of this polymer and devices comprising polylactide.[37] Conversely, PTMC does not undergo instant degradation but EB irradiation causes both degradation and intermolecular cross-linking occurring simultaneously. Irradiation of PTMC with doses sufficient enough results in an increase in its molecular weight and eventually in formation of insoluble fraction – gel.[21] In the present investigations, reduction of molecular weight of PLLA and nearly unaffected molecular weight of PTMC were observed for polymers irradiated separately by EB with a standard sterilization dose of 25 kGy – Table I. Also, averaged molecular weight of the blend of these two polymers was measured before and after application of EB sterilization. Examination of the polymer blend resulted in a single broader peak in GPC trace, the M_n and M_w molecular weights were in between those of their parental polymers but polydispersity index (PD) was larger. Reduction in averaged molecular weights and further increase of PD after irradiation was observed.

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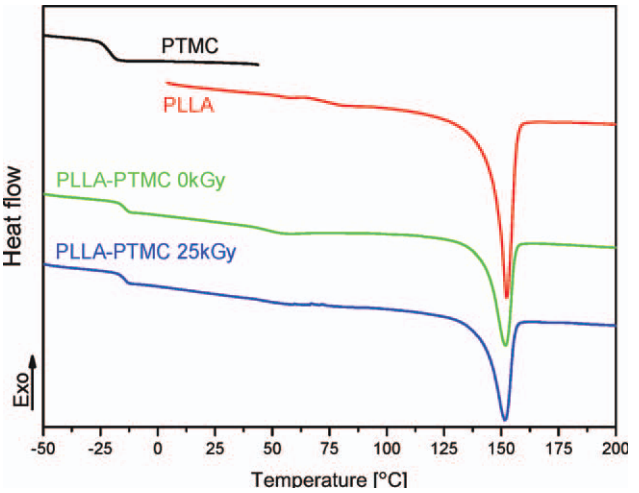

Figure 4. Traces of the first heating scan for PTMC, PLLA, the PLLA/PTMC blend and the blend after EB sterilization with 25 kGy. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Besides molecular weight, thermal characteristic of the blend was evaluated – traces of first heating scan are presented in Figure 4, and the thermal transitions data is collected in Table II. Two glass transitions temperatures (based on the second heating scan) at about −14 to −18 and 60°C were detected, and they are assigned to PTMC and PLLA T_g's, respectively. Additionally transition related to PLLA crystalline phase melting (first heating scan) was observed at about 152°C. Presence of ordered structure of PLLA chains in the blend indicates that the mixing process of PLLA and PTMC was not absolute, and some clusters of only one or another polymer remained in the product. Partial crystallization may take place even in PLLA/PTMC block and random copolymers samples.[38,39] Table II shows measured values also for irradiated material. The transition temperatures remained unchanged after application of the sterilization dose but the DC of PLLA increased. Therefore, we can assume that, in spite of the degradation or crosslinking reactions that is related to molecular mass change, the chemical composition of macromolecules was retained or at least the changes are minor. Indeed, chemical structures of PTMC[21] and PLLA[40,41] subjected to 25 kGy, and evaluated by other physicochemical methods, were reported to be nearly unaffected.

Tensile testing experiments were undertaken to evaluate the impact of the polymers molecular weight reduction on toughness of the guidance tubes. An example of the stress–strain curve is presented in Figure 5(a). First of all one should bear in mind that the tube wall has a porous structure that enhances flexibility that was already provided, to some extent, by elastomeric polymers. In contrast, porosity within the walls is the cause of some reduction of tensile strength as compared to solid polymer blend of PTMC and PLLA. Evaluation done in our laboratory showed that tensile stress measured for PLLA/PTMC (1 : 1 wt/wt %) film casted from common solvent was about 4 and 2 MPa for unsterile films and that irradiated with 25 kGy, respectively,[42] that is only slightly higher than that measured for the tube Figure 5(b). The strength of the current guides is somewhat superior to that reported for tubes fabricated from PLLA-PTMC copolymer, that is, about 0.5 MPa.[43] Those scaffolds were obtained by immersing a mandrel in copolymer solution containing also a porogen particles, which were subsequently leached out, resulting in high porosity of the walls.

Nevertheless, when implanting the tubes into animals a minimum strength that can withstand handling and suturing is necessary. In the intended application, two stumps of broken nerve will be introduced into two ends of the tube and sutured in with one stitch at each side. As examined by surgeons who are going to perform implantation (the animal experimentations involving rats are in the last preparation stage – the Local Ethical Committee had allowed for the trials) the tubes are sufficiently strong and enough flexible to be applied for animals without any adverse effects such as unexpected fracture of the tube or its kinking that may cause an injury of surrounding tissue and malfunctioning of the device. To be precise, tensile strength of a single tube is of about 15 N regardless to MC presence. Some difference in mechanical properties was detected after EB sterilization, that is tensile strength and maximum strain were reduced, whereas Young's modulus remained constant as compared in Figure 5 (gray bars of PLLA/PTMC – 0 and 25 kGy). Obviously, degrading PLLA component controls mechanical strength of the tubes.

Admixture of MC to the synthetic polymers may result in variation in some properties, and therefore, influence performance of the tubes. Moreover, sterilization accomplished by EB irradiation could have an impact as well. Besides several reports on the evidences of cross-linking of polysaccharides derivatives[44,45] those materials of natural origin tend to degrade when subjected to ionizing radiation in solid state. Behavior of MC exposed onto EB irradiation is similar.[46] Application of 25 kGy of ionizing radiation for MC in solid state reduces viscosity of its solution of about 50%, which corresponds to significant

Table II. Thermal Characteristics of PTMC, PLLA, and Tubes Made from Blend PLLA/PTMC and Blend with MC, Nonsterile, and Sterilized with EB of 25 kGy

Polymer	T_g I (°C)	T_g II (°C)	T_m (°C)	ΔH (J/g)	DC[a]
PTMC nonsterile	−18.6				
PTMC 25 kGy	−18.0				
PLLA nonsterile		60.2	150.6	33.0	30.9
PLLA 25 kGy		59.7	150.1	33.8	31.7
PLLA/PTMC nonsterile	−14.3	58.9	151.9	18.2	38.9
PLLA/PTMC 25kGy	−14.3	60.1	151.7	20.5	43.8
PLLA/PTMC/MC nonsterile	−13.5	59.3	152.4	16.5	39.2
PLLA/PTMC/MC 25 kGy	−13.8	59.9	151.7	22.8	54.2

Glass transition temperature (T_g) recorded at the second heating scan, and melting enthalpy (ΔH) and melting temperature (T_m) measured at the first heating scan.
[a]DC – degree of crystallinity of PLLA fraction.

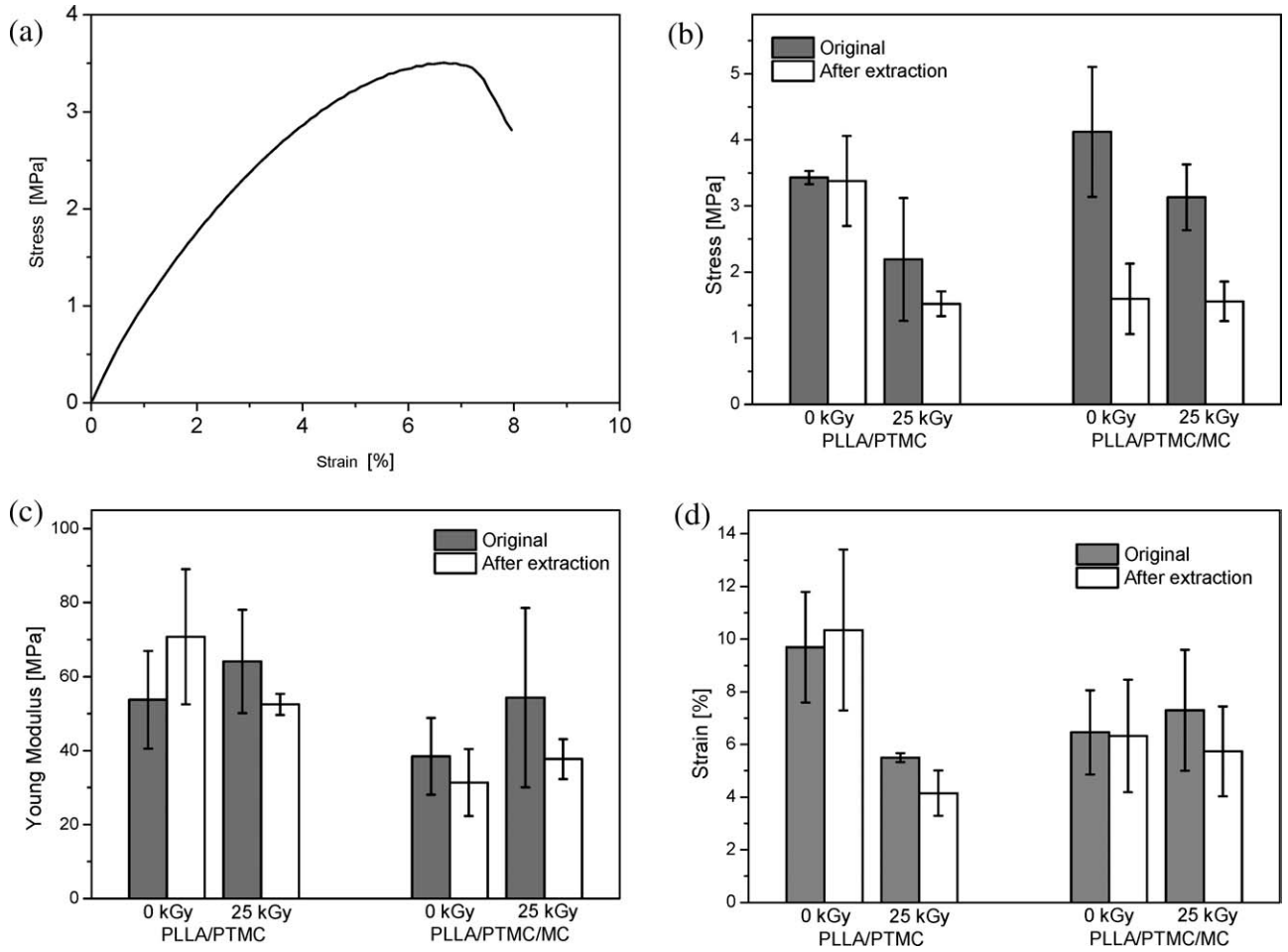

Figure 5. Tensile testing of guidance tubes of PLLA/PTMC and PLLA/PTMC/MC unsterile (0 kGy) and sterilized with EB (25 kGy), before and after 10 days extraction – gray and white bars, respectively. (a) example of stress–strain profile for PLLA/PTMC nonsterile tube, (b) maximum stress, (c) Young Modulus, (d) strain.

decrease in its molecular weight.[47] This feature of MC is taken under account when conducting sterilization by ionizing radiation, but the effect of MC degradation on overall functioning of manufactured tubes seems to be minor. Therefore, no evaluation particularly devoted to characterization of MC degradation was done within a framework of the present studies.

Admixture of MC had minor impact on the tensile properties of the tubes. As can be seen in Figure 5 (compare 0 kGy grey bars) there is no significant difference for any measured parameters between samples with and without MC and the impact of sterilization is also comparable for both kinds of tubes. The only parameter that decreased somewhat for samples containing MC is the strain, nevertheless there was no further change caused by sterilization. Presence of MC chains or clusters within the matrix of synthetic polymers may reduce the strain due to the fact that MC is a brittle material.

As the product of nerve guidance tube will be in constant contact with body fluids and its composition is biodegradable polyesters/MC blend, one can expect that water-soluble MC might be easily dissolved and extracted from the insoluble polyester matrix. To confirm such phenomenon sterilized and nonsterile tubes comprising PLLA/PTMC polymers with and without MC were immersed in PBS solution for 10 days with gentle shaking and subsequently mass loss of the tubes and their tensile properties were examined.

Extraction of water-soluble polysaccharide had an impact on morphology and mechanical properties of the tubes. Primarily, changes of mass of the tubes were evaluated. Mass of those comprising MC decreased of about 7–8 wt % after 10 days extraction, as can be seen in Figure 6. It corresponds to nearly entire MC in the sample, based on the polymer fractions in the solution during immersing of a mandrel, which was about 9 wt %. Moreover, based on this evaluation we estimate that during manufacturing process, specifically during washing formed tubes in water and alcohol, some of the MC, that is, up to 20% of its mass was removed. Mass of tubes without the polysaccharide, that is, only PLLA/PTMC constituents did not changed after extraction. Biologically active molecules commonly used in nerve regeneration assistance for stimulation of nerve growth, that is, growth factors are proteins with molecular weight of several tens kDa (kg mol^{-1}). Thus, based on the aforementioned result of MC extraction, we can expect that also water-

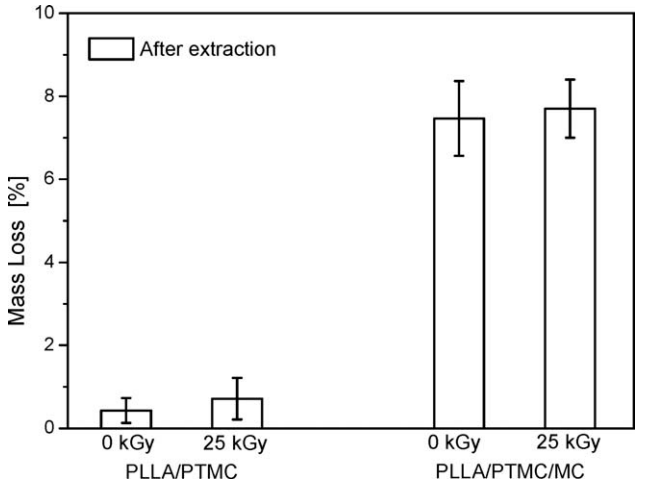

Figure 6. Mass loss of guidance tubes of PLLA/PTMC and PLLA/PTMC/MC, unsterile (0 kGy) and sterilized with EB (25 kGy) after 10 days extraction.

soluble proteins can be easily released if immobilized in the synthetic polymers matrix instead of the polysaccharide. It is known that certain growth factors should be delivered to the restoring nerve within short period after the lesion occurred.[48] Therefore, the tubes in current form may be suitable as a medium releasing growth factors directly to the environment of regenerating nerve.

Molecular weight and thermal characteristic of the synthetic polymers after MC extraction were determined as well, and the results shown that they remained unchanged as compared to the material originated from the tube without MC. SEM picture presented in Figure 3(d) taken after 10 days extraction revealed pores of similar size but of slightly higher dispersion, thus, some changes in mechanical properties were anticipated. Indeed, after extraction the tensile strength of samples with MC was reduced to about half of its initial value (Figure 5 PLLA/PTMC/MC), whereas this did not change for the tubes made of synthetic polymers without MC (Figure 5 PLLA/PTMC). There was no difference in modulus nor in strain measured for tubes made of those two compositions. This phenomenon is expected to have no significant impact on tubes performance after implantation, since tension is generated mostly during operation, for example, suturing in and immediately afterwards, then, it is reduced instantly due to relaxation of the nerve itself and surrounding tissue that adjusts to the new conditions.

Cytotoxicity Evaluation

Preliminary biocompatibility study of nerve regeneration tubes was done by two indicative test for assessing potential cytotoxicity of material, that is LDH and MTT assays. LDH cytotoxicity assay is based on the measurements of cytoplasmic enzyme activity released from damaged cells. The amount of enzyme activity correlates to the proportion of damaged cells number. Incubation of samples in the medium that was consequently evaluated with LDH assay was performed for 24 h. The results of LDH tests are presented in Figure 7(a). In the applied test, Triton X-100 was used as a positive control, which causes 100% cells death. The results for investigated samples and negative controls – polyethylene and cells incubated only with medium – showed that those materials had not triggered death of cells, what means that none of the tested materials was cytotoxic.

MTT test is a sensitive *in vitro* assay for the measurement of cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability is observed. Results of MTT test after 48 h of samples incubation are presented in Figure 7(b). Results for a negative control, that in this case was Triton X-100 indicates 100% reduction in overall cell viability – the cells that were in contact with this toxic agent did not survived. Investigated materials showed good biocompatibility while contacted with cells. Cell viability number was not lower than that for positive controls, that is, cells incubated only with medium and cells with polyethylene. The results of both *in vitro* tests showing no cytotoxic effects on cells allowed us to consider *in vivo* experimentation using small animals, primarily subcutaneous implantation, for evaluation material interaction with living tissue, followed by femoral nerve regeneration studies.

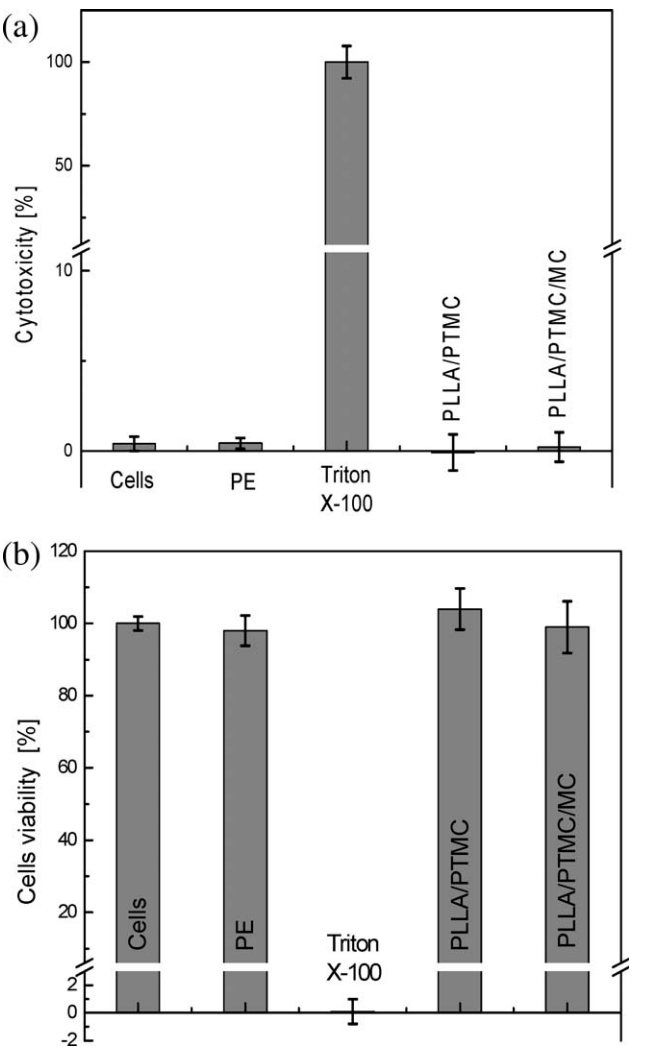

Figure 7. Results of cytotoxicity tests conducted for two different tube materials sterilized by EB irradiation at 25 kGy: (a) LDH – 24 h incubation and (b) MTT – 48 h incubation.

CONCLUSION

Present investigations related to manufacturing of nerve guidance tubes were initiated in response to clinical needs. Application of a tubular scaffold that is implanted into lesion has potential advantages over traditional methods, therefore, biomaterials comprising biodegradable polymers with appropriate physicochemical properties were developed. Sufficient tensile properties, flexibility, and resistant to fracture by a thread during suturing in were achieved for selected polymer composition and manufacturing conditions. Polymer that is responsible for the strength, PLLA and rubber-like PTMC were formed into tubes by phase inversion process of the polymers in solution deposited on a mandrel when contacted with a nonsolvent. This resulted in porous structure, that was additionally modified with water-soluble polymer of natural origin, MC. Moreover, MC performed as a model or carrier for biologically active compounds, for instance growth factors, that may be released within several days after implantation. Results of the physicochemical evaluation and cytotoxicity testing showed good suitability of guidance tubes for potential exploitation in nerve regeneration. Lack of inacceptable cytotoxic effect of polymeric materials of PLLA, PTMC, and MC opens the way to apply manufactured guidance channels for *in vivo* testing, that has been already initiated and the outcome will be published as soon as the results are available.

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