BIOCOMPATIBILITY OF L-LACTIDE-CO-GLYCOLIDE-CO-TRIMETHYLENE-CARBONATE SHAPE MEMORY TERPOLYMER WITH HUMAN CHONDROCYTES

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Abstract

Biomedical application of biodegradable shape memory terpolymers obtained from L-lactide, glycolide, trimethylene carbonate (TMC) have been intensively investigated in recent years. All applicable biomedical materials must be biocompatible, which means that they cannot cause toxic, cytotoxic, allergic, or carcinogenic reactions. The first study evaluating the biocompatibility of the material is determination of its cytotoxicity.

The purpose of this study was to assess the biocompatibility of poly(L-lactide-co-glycolide-co-trimethylene-carbonate) 75:13:12, synthesized using $Zr(Acac)_4$ as an initiator of polymerization. The terpolymer was degraded for 30, 60 and 90 days at 37şC in water. The effect of degradation products on the growth of human articular chondrocytes was determined using the sulforhodamine B assay. The examined terpolymer was characterized by means of NMR spectroscopy, GPC and DSC. The results showed that the studied terpolymer was biocompatible with tested cells.

Keywords: *Shape-memory polymers (SMP), biodegradable polymers, cytotoxicity, degradation products, in vitro, biocompatibility of terpolymer*

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Introduction

Shape memory effect is an ability of material to return from a temporary shape, which is obtained as a result of mechanical deformation, to the permanent (primary) shape, as a result of stimulation by various factors - physical (such as temperature, light (UV), IR radiation, magnetic or electrical field); or chemical (such as changes of pH, ionic strength etc.) [1,2].

The first materials used in medicine due to shape memory effect were metal alloys (SMA - shape memory alloys). The most commonly and most widely used is Nitinol - alloy of nickel and titanium. However, the SMA have a lot of disadvantages and because of this, researchers are still seeking for new innovative materials with shape memory effect. Nowadays, the most commonly used and studied materials are SMP - shape memory polymers. This is a group of polymers demonstrating the ability of shape memory effect. The SMP when compared to SMA have many advantages e.g. lower manufacturing cost, the ability to recover the primary shape in expected return temperature (wide temperature range), and also SMP are much easier to process (easier production of various shapes and forms) [1,2].

Biodegradable materials derived from L-lactide, glycolide and trimethylene carbonate (TMC) are the subject of many studies in recent years. This is related with their biocompatibility and degradation to the nontoxic products of metabolism. They were already used in medicine as surgical sutures, scaffolds for tissue engineering or controlled drug delivery systems. Terpolymers derived from these monomers may have shape memory effect, which further increases their potential biomedical application as, for example, self-locking surgical staples, self-expanding stents etc. [3-5].

Regardless of the specific biomedical application, each new material must meet several basic features, including the most important – biocompatibility. This is the total compatibility of the material with the human body which is lack of toxic reactions, cytotoxic agents, allergic reaction or carcinogenic effects. First study, allowing for an initial evaluation of biocompatibility of the material, is a cytotoxicity test [6].

Cytotoxicity of the polymeric material may be affected by many factors – monomeric composition, molecular weight of the polymer, the weight of the final product, its structure and shape (the presence of sharp edges) and also the rate of degradation of the material [6,7]. Crucial importance may also have the residues from synthesis of polymer, released from the material as a result of its degradation - the initiator, solvent or trace amounts of other contaminants [8,9]. Monomeric composition determines the generated degradation products. The final products of degradation of polyesters as PLA, PGA, PCL or their copolymers are lactic acid, glycolic acid and 6-hydroxyhexanoic acid [10,11]. Release of these substances in large quantities and in short time can lead to a reduction in local pH (at the site of implantation of the material) and may induce inflammatory reaction [8]. This problem may be solved by using polymeric materials which degrade to inert products - good examples are the polymers derived from trimethylene carbonate which primary product of the degradation is 1,3-propanediol [12].

The terpolymer used in this paper was synthesized with zirconium acetylacetonate $Zr(Acac)_4$ as an initiator. Stannous octoate (II) ($Sn(Oct)_2$) is commonly used as an initiator of the polymerization reaction [8], but because of its toxicity, researches are looking for other, non-toxic initiators (such as calcium, iron or zirconium) [13]. Studies have shown that the use of $Zr(Acac)_4$ as the initiator have positive effect on the viability and activity of cells grown on a polymer (compared to polymers containing trace amounts of tin) [8].

The aim of this study was to evaluate the cytotoxicity of degradation products derived from poly(L-lactide-co-glycolide-co-trimethylene-carbonate) 75:13:12, on cultured human chondrocytes.

Materials and methods

The polymeric matrices were obtained from poly(L-lactide-co-glycolide-co-trimethylene carbonate) (P(L-LA-GA-TMC)) 75:13:12. The terpolymer was synthesized at the Centre of Polymer and Carbon Materials of Polish Academy of Sciences in Zabrze. Terpolymerization reaction was conducted in bulk at 120°C for 72 h, with the use of non-toxic $Zr(Acac)_4$ as an initiator.

Composition of terpolymer was examined with the use of 1-H NMR high resolution spectroscopy (AVANCE II Ultra Shield Plus, Bruker 600 MHz) with $CDCl_3$ as a solvent.

TABLE 1. Characteristic of P(L-LA-GA-TMC), (F_T, F_{LL}, F_{GG} - the percentage content of carbonate, lactidyl and glycolidyl units, respectively; M_n – number average molecular weight, D – molecular-weight dispersity M_w/M_n), T_g – glass transition temperature, T_m – melting temperature, ΔH_m – melting enthalpy).

	F_T	F_{LL}	F_{GG}	M_n [Da]	D	T_g [°C]	T_m [°C]	ΔH_m [J/g]
P(LA-GA-TMC)	12	75	13	39400	2.2	52	120	8

TABLE 2. Characteristic of P (L-LA-GA-TMC) matrices after 30, 60 and 90 days of degradation.

	F_T	F_{LL}	F_{GG}	M_n [kDa]	D	Weight loss [%]
0 days	12	75	13	39.4	2.2	-
30 days	14	74	12	11.9	3.4	4.5
60 days	14	74	12	2.6	6.4	6.1
90 days	14	74	12	2.1	5.2	7.4

Thermal properties were tested by differential scanning calorimetry (DSC) (TA DSC 2010; TA Instruments, New Castle, DE). Terpolymer was also characterized by number average molecular weight (M_n) and molecular weight dispersity (D) that were analyzed by means of gel permeation chromatography (Viscotek RImax; Viscotek 3580 columns), with chloroform as eluent and 1mL/min flow rate, based on polystyrene standards.

Matrices used in this study, were obtained by the compression method. The steel frame (area of 40.2 cm^2) was placed under a hydraulic press and it was filled with appropriate amount of polymer (4.2 g). The compression occurred in elastic state of terpolymer (above T_g) between heated stainless steel blocks with pressure of 2.3 tons for 4 minutes. The film was cut in order to prepare 1cm diameter matrices. The matrices were sterilized with high-energy electron beam radiation in dose of 25 kGy .

In this study matrices of terpolymer in the form of discs were placed in sterile nonpyrogenic water (Hyclone, Thermo-Scientific) in test tubes with corks. The volume of degradation medium was dependent on the mass of the polymer disc and calculated by the following formula:

Water volume = 25 x matrix mass (g).

Then, the matrices were placed in an incubator at 37°C. The matrices degraded in water during 30, 60 and 90 days (ISO 10993-13 standard), then they were removed and aqueous solutions of degradation products were tested using the cytotoxicity test. Pure water was used as a negative control.

The first step of the testing of aqueous solutions was to prepare appropriate medium for the test. The culture medium was prepared using the basic medium: MEM concentrated x10 (Minimum Essential Medium Eagle's, Sigma). The concentrated medium was diluted with sterile, ultrapure deionized water, added in suitable proportions to obtain dilution x1. For the enrichment of the basic medium, following supplements were added:

- 10% fetal bovine serum (FBS) (PAA)
- penicillin 100 U/ml, streptomycin 100 g/ml (Sigma)
- AAMS supplement containing amino acids (Sigma)
- Glutamine to final concentration of 2 M (Sigma)
- $NaHCO_3$ a final concentration of 2 /l (Sigma)
- ITS supplement (insulin, transferrin, selenium, Invitrogen)
- HEPES at a final concentration of 10 M (Sigma)

Cytotoxicity test was performed on normal human articular chondrocytes (Lonza). The cells were cultured in Chondrocyte Growth Medium (CGM BulletKit, Lonza) under standard conditions (at 37°C, in the atmosphere of 5% CO_2 / 95% air).

For the assessment of the cell proliferation, chondrocytes were seeded into 96-well plates (10^3 cells/well in 0.2 ml of CGM). After 24 hours of incubation, CGM medium was removed from wells, and prepared aqueous solutions (in two dilutions: 1:4 and 1:20 with medium as described above) were added. The cells were incubated with solutions for 3 days.

Cell proliferation was quantified with the use of In Vitro Toxicology Assay Kit, Sulforhodamine B Based (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, cells were fixed with TCA (trichloroacetic acid) and then stained with sulforhodamine B, which is a dye staining cellular proteins. The unbound dye was removed with 1% acetic acid and the incorporated dye was liberated from cells with Tris solution. Absorbance was measured at λ = 570 nm and λ = 690 nm (reference wavelength).

The last part of this study was statistical analysis of the results. Analysis was performed using Statistica 8, with quantity tests: normality tests, Leven's and Brown-Forsythe's variance tests, variance analysis (ANOVA) and RIR Turkey's test ($p<0,05$).

Results and Discussion

The aim of present study was to assess the biocompatibility of degradation products of biodegradable polymer with the shape memory property. Terpolymeric matrices were obtained from poly(L-lactide-co-glycolide-co-trimethylene carbonate) (P(L-LA-GA-TMC)) 75:13:12, which was synthesized with $Zr(Acac)_4$ as an initiator of terpolymerization and processed by pressing. The characteristic of the material used in this work is shown in TABLE 1.

The analyzed P(L-LA-GA-TMC) 75:13:12 is a material with a high number average molecular weight (M_n) and low molecular-weight dispersity (D). The glass transition temperature of the polymer (T_g), determined by DSC was 52°C and T_m = 120°C, however the melting enthalpy was very low. The changes of polymer composition, molecular weight and thermal properties after 30, 60 and 90 days of degradation were shown in TABLE 2. Significant decrease of M_n was noted between 30 and 60 days of degradation, however the weight loss was not significant. Faster degradation of lactidyl and glicolidyl units was determined after 30 days of degradation. Between 30 and 90 days of degradation the terpolymer composition did not changed, so all the units degraded evenly.

The results of cytotoxicity test of studied aqueous solutions of degradations products are shown in FIG. 1.

Aqueous solutions of the degradation products of P(L-LA-GA-TMC) at a dilution of 1:4 showed statistically significant inhibition of growth of chondrocytes only in samples obtained after 60 days degradation. The lack of cytotoxicity of the samples obtained after 90 days of degradation (despite the cytotoxicity of samples which degraded only 60 days) may show that the factor which was toxic to the cells, after an additional 30 days of degradation also degraded.

Aqueous solutions of the degradation products of P(L-LA-GA-TMC) at a dilution of 1:20 did not cause any statistically significant inhibition of growth of the studied cells – this may be caused by higher dilution of cytotoxic agent.

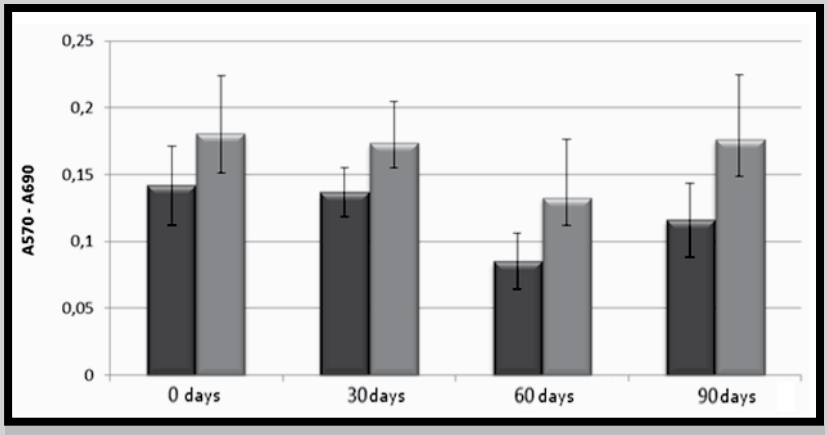

FIG. 1. Influence of water solutions on human chondrocyte growth. Bars show the average value of absorbance levels (±SD), n=9, p<0.05.

The cytotoxicity of these materials may be provoked by many factors. According to the literature [8], degradation products can provoke cytotoxicity if they have low molecular weight. There are also studies that show inflammatory reaction in correlation to the decrease of M_n of 10 – 0.5 kDa and undoubtedly inflammation has negative effect on tissue and cells [8]. Secondly, other factor that may be responsible for cytotoxicity is local pH decrease that may be provoked by acidic degradation products released from the material. According to Cordewener et al. [8] there is a strict correlation between pH decrease and toxicity effect. Finally, processing method may also be a factor affecting cytotoxicity. In this study, the terpolymeric matrices were obtained by compression method. The study of Chłopek et al. [14] shows that poly (L-lactide-co-DL-lactide) 70:30 changes its properties observed in biological environment depending on the processing method. Compression method may affect polymeric material and cause faster degradation, which may more significantly decrease pH of degradation medium. In this study, the effect of pH decrease was eliminated during preparations of the samples by adding of appropriate amounts of NaOH.

It should be noted that even lack of cytotoxicity is not sufficient to conclude that the material is completely biocompatible. However, such a result is required for further research in this aspect [6]. The results of this study suggest that the studied terpolymer (P(L-LA-GA-TMC) 75:13:12) is biocompatible, which should be further examined. It is not cytotoxic during first 30 days of degradation. After this time the degradation products obtained from P(L-LA-GA-TMC) show cytotoxic effect on human chondrocytes. Further studies of P(L-LA-GA-TMC) are needed to determine the cytotoxic factor; compare biocompatibility of polymer with the same composition but with higher molecular weight, or obtained by modified processing method.

Conclusion

Based on the results of this study, there was inhibition of growth of chondrocytes incubated with degradation products obtained from matrices of P(L-LA-GA-TMC) 75:13:12. However, terpolymer does not show cytotoxic effect to chondrocytes during the first 30 days of degradation.

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