

Fabrication, properties and cytotoxicity evaluation of degradable poly(trimethylene carbonate-co-lactide) for the use as nerve guidance channels

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Abstract

Strategies to improve healing of damaged nerves include the application of specialized nerve guides, which hold the promise for allowing reanastomosis of the severed or damaged fibers. Studies have demonstrated that the use of a slowly degradable polymeric nerve guide can improve the nature and rate of nerve regeneration across a short gap in small nerves. The objective of this study was to characterize a biodegradable nerve guide based on poly(trimethylene carbonate-co-lactide) for peripheral nerve regeneration and to evaluate its cytotoxicity. The obtained copolymer films were incubated in two different media (distilled water and simulated body fluid), and while the degradation process appeared, pH and ion conductivity changes of solutions were monitored as well as mass loss of the samples. Additionally, mechanical tests (tensile strength, elongation at break and Young's modulus parameters) before and after different time points were carried out. To evaluate cytotoxicity biological test were done on fibroblasts cells (NIH 3T3). Cell metabolic activity was determined using Alamar Blue reagent and their morphology was observed under fluorescence microscopy. The growth of pH in both media were mostly caused by steadily degradation of carbonate units into alkaline diols. The growth of ion conductivity value at the beginning of the incubation process was associated with the releasing of free ions to the solution. The mechanical parameters decreased with the progress of degradation process. Ringer's fluid, as more aggressive, caused higher decrease in mechanical properties. The measured contact angles showed good surface wettability. Both surfaces, the top and the bottom, had similar hydrophilicity. Moreover, activity of fibroblasts cells were similar on both sides as well as on the reference TCPS. Good adhesion of NIH 3T3 cells to the surface suggests that the hydrophilic polymers promote colonization of fibroblasts cells on their surface. Biological studies have shown that used cells are very sensitive to surface topography which they colonize and cell viability was higher at the bottom surface, which has a slightly higher average roughness R_a . Thus, fibroblasts cell preferred colonizing rougher than smoother surfaces. Fabricated films does not affect negatively, namely, toxic on cell cultures and forms substrate with favourable surface properties. This was confirmed by the Alamar Blue tests and microscopic observations.

Key words: peripheral nerve regeneration, degradable polymer, PTMC/PLA, contact angle, roughness, morphology, viability, fibroblasts

Introduction

The current clinical method for treatment of nerve gap is reconstruction with the use of autologous nerve grafts. However, autografting is limited by the availability of expendable donors nerve and donor site morbidity [1]. An alternative repair method is tubulization, which involves enclosure of the end of severed nerve by a tube which holds the stumps in correct place, offers a guide to regenerating axons to distal stump and may concentrate neurotrophic products from nerve stumps [2, 3]. The use of synthetic conduits as nerve guides to bridge missing tissue has provided an excellent in vivo experimental model to study peripheral nerve regeneration process. Of five channels approved by Food and Drug Administration (FDA), four are based on degradable polymers and of these, two are composed of collagen and two are synthetic, composed of poly(glycolic acid) [4] and poly(lactide-co-caprolactone) [3, 5-9]. Biodegradable nerve tubes are advantageous over their non-degradable ones, obviating the need for their removal when regeneration is completed. These temporary conduits have been mostly based on aliphatic polyesters including poly(glycolic acid) [10-12], poly(lactid-co-glicolid acid) [13-17], poly(DL-lactic acid) [18-20] and poly(lactic acid-co-ε-caprolactone) [5,21]. The aforementioned group have disadvantage of not only being stiff and brittle materials but also of possessing a relatively high rate of degradation. Additionally, high degrees of swelling are observed at late stage of degradation [22, 23]. In view of desired properties of synthetic tubes, copolymers of trimethylene carbonate (TMC) and lactides are suitable candidate for designing peripheral nerve implants, as both polymers undergo slow degradation in vivo to non-toxic products [24,25]. Poly(trimethylene carbonate) (PTMC) belongs to the family of poly(alkylene carbonate)s and is obtained through polymerization of its cyclic monomer, trimethylene carbonate. It is a linear, amorphous polymer

with glass transition of approximately -15 °C. At room temperature it is a rubbery, flexible material [26-30]. PTMC seems to be an interesting candidate to introduce modifications to rigid PLLA [31-34]. Poly(L-lactic acid) (PLLA) is a well-known polymer that has been studied extensively for various biomedical applications owing to its acceptable biocompatibility and inherent biodegradability. PLLA, an example of aliphatic polyesters commonly made from α-hydroxy acids is a thermoplastic, high-strength and high-modulus polymer. The rate of degradation of PLLA depends on factors such as its initial molecular weight, purity, crystalline morphology, the isomer ratio and external dimensions of a device [35-40].

The aim of this study was to investigate the influence of poly(trimethylene carbonate-co-lactide)'s degradation process in two different incubation liquids on mechanical properties as well as to evaluate cytotoxicity of PTMC-PLA and to assess the influence of surface parameters of the PTMC/PLA on fibroblasts adhesion and proliferation.

Materials and Methods

Materials

A copolymer of trimethylene carbonate and L-lactide (PTMC-PLA, 85:15) was purchased from BiomatPol (Poland) and its chemical structure is presented in Fig. 1. It was synthesized by ring opening polymerization of L-lactide and 1,3-trimethylene carbonate, in the presence of low-toxic initiator $Zr(acac)_4$ – at a molar ratio of 1.25×10^{-3} at 100 °C by a conventional method using a vacuum line for degassing and sealing of the ampoules.

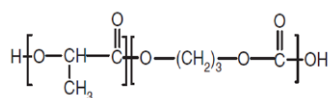


Figure 1. Chemical structure of copolymer PTMC-PLA

Preparation

Films were prepared by dissolving PTMC-PLA polymer in dimethylformamide to obtain a 10 wt. % solution. The solution was stirred with magnetic stirrer for at least 48h at ambient temperature and then cast on glass Petri dishes. The films were dried under vacuum for 48 hours. For biological evaluation the films were sterilised with the use of the H₂O₂ cold plasma technique (Sterrad, ASP, J&J, USA).

In vitro degradation

For each degradation studies PTMC-PLA samples were placed in small flasks filled with distilled water or Ringer's fluid. The flasks stood in the thermostatic oven at 37°C for specified period of time. After preterminated time of incubation samples were removed from the solution and dried under vacuum. The ratio of samples' mass to incubation medium for every flask was constant. The hydrolytic degradation process was observed by pH, conductivity and mass loss changes. The measurements were carried out with pH-meter/conductometer CPC-411 (Elmetron, Poland) and mass loss were done using balance WAS 220/C/2 (Radwag, Poland) according to formula 1. Subsequently samples were evaluated by several physicochemical methods.

$$\Delta m = \frac{m_0 - m_1}{m_0} \times 100\% \quad (1)$$

where:

Δm - mass loss [%]

m_1 - residual mass of sample [g]

m_0 - initial mass of sample [g]

Determination of physicochemical properties of PTMC-PLA

The water contact angle of obtained PTMC-PLA films was measured using sessile drop method on Drop Shape Analysis System (DSA Mk2, Krüss, Germany). Ten measurements on the film were ac-

complished. The data presented are average of ten measurements (\pm standard deviation).

Prior to taking roughness measurements a glass slide with the thin PTMC-PLA films was fixed onto a mount with double-sided adhesive tape to prevent the samples from moving during the test. Roughness was measured with using a profilometer (Hommelwerke, Germany), equipped with cone shaped diamond tip (radius of 5 μ m) and the velocity of moving cone was 0,50 mm/s. Each sample was measured ten times on both sides. The parameters calculated were surface average roughness (R_a). All the given values are presented as average of ten measurements (\pm standard deviation).

Tensile strengths (TS), Young's moduli (E) and elongation at break (ϵ) of samples (70x5) were measured using a universal testing machine (Zwick 1465, Germany) equipped with 1kN load cell. The sample length between the clamps was 45 mm and clamps' speed was 50 mm/min. The obtained results correspond to the average of six measurements (\pm standard deviation).

Determination of biological properties of PTMC-PLA

The sterilized films were placed in 24-well plate (Nunc, Denmark), both surfaces of the films (top surface, i.g. air-cured, and bottom surface, i.g. glass-cured) were tested and as a control the bottom of the well tissue culture polystyrene-TCPS was used. NIH 3T3 mouse embryonic fibroblast cells were cultured on the studied materials in EMEM (Eagle's minimal essential medium) cell culture medium (PAN Biotech, Germany) supplemented with 10% FBS, 1% penicillin/streptomycin and 0.1% amino acids and sodium pyruvate (PAA, Germany) at 37°C under a humidified atmosphere with 5.0% CO₂ for 24 hours, 4 and 7 days. Initial cell density was 2.5×10^4 cells per well.

Cell metabolic activity was evaluated using Alamar Blue reagent (In Vitro Toxicology Assay Kit, Resazurin based). 0.1 ml Alamar Blue rea-

gent was added and the cells were incubated for 4 h at 37°C. Reduction of Alamar Blue was measured fluorescently (excitation wavelength 530 nm, emission wavelength 590 nm) (FLUOstar Omega, BMG labtech) and calculated according to the following formula:

$$\% \text{Reduction of Alamar Blue} = \frac{S^x - S^{\text{control}}}{S^{100\% \text{ reduced}} - S^{\text{control}}} \cdot 100\% \quad (2)$$

where:

S^x – fluorescence of samples

S^{control} – fluorescence of medium without cells

$S^{100\% \text{ reduced}}$ – fluorescence of reagent reduced in 100%

The result of this measurement is the reduction ratio of the reagent (the higher the reduction, the more cells).

The results were expressed as mean and standard error of the mean from three independent samples. Statistical significance was evaluated according to t-test.

Morphology of the cells was observed under fluorescence microscopy (Zeiss Axiovert, Carl Zeiss, Germany). The cultured cells were fixed in 4% paraformaldehyde for 1 h, then washed in PBS and stained with acridine orange solution (1 mg/mL) to visualize the nucleic acids.

Results and Discussion

In order to design a suitable system for the preparation of nerve guidance channels to be used in regeneration of nerve gaps many limitations are imposed. Namely, slow degradation *in vivo*, low degree of swelling after implantation and no or low crystallinity. Moreover, implant should be flexible and tough to allow the handling in microsurgery. Therefore, polymer system should meet several requirements in terms of mechanical performance, suitable surface properties enhancing cells activity, degradation behavior and lack of cytotoxicity.

The degradation kinetics of neural guidance should match the rate of nerve regeneration. Too rapidly occurring degradation may result in failing to protect regenerated axons and too slow degradation rate may lead to compression and foreign body reaction. Degradation process play crucial role in nerve regeneration and thus it was investigated in both distilled water and Ringer's fluid, presented in figure 2.

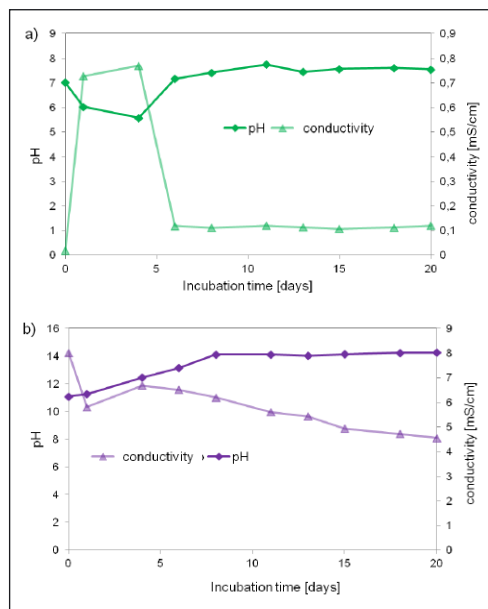


Figure 2. Solution values of pH and conductivity of PTMC-PLA films incubated in a) distilled water and b) Ringer's fluid

It is clearly visible that at first pH of PTMC-PLA films incubated in distilled water decreased to 5.58, suggesting that lactide units (PLA) decomposes to oligomers and lactic acid faster than carbonate units (PTMC). However, after a 4 day pH increased with single slight fall. Further increase might result from degradation of PTMC units, which decomposed to alkaline products i.e. 1,3-propanediol. The ionic conductivity was in inverse proportion to pH changes. In the initial step of incubation it rapidly increased to approx. 0.73 mS/cm, which was connected with releasing of free ions to the solution and after 4 day main-

tained almost constant at 0.12 mS/cm. Unlike the samples' degradation in water environment, films incubated in Ringer's fluid showed no decrease of pH in first step of degradation, presented in figure 2b. The pH values increased gradually, which was due to constant decomposition of TMC units to alkaline products. Figure 3 presents the mass loss during incubation in both distilled water and Ringer's fluid. The PTMC-PLA samples lost their weight steadily without the significant changes and finally reached 2.86%. Whereas films incubated in Ringer's fluid showed higher rate of degradation process and amounted to 5.57%.

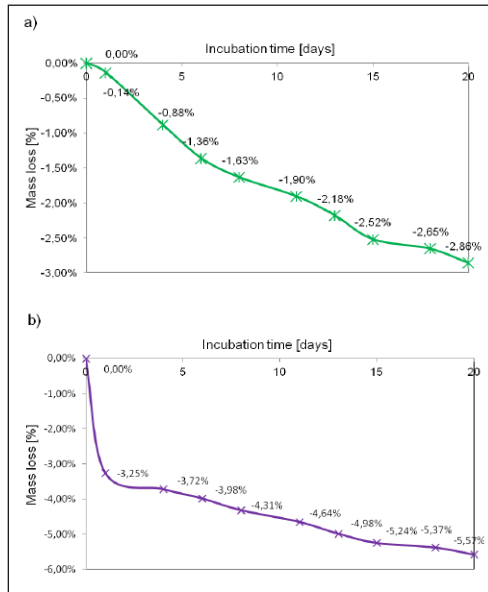


Figure 3. Mass loss of PTMC-PLA films incubated in a) distilled water and b) Ringer's fluid

During nerve regeneration, peripheral nerve implants should have the capacity to withstand mechanical stress from neighbouring tissues and maintain at least slight elasticity and bendability without collapsing or losing their shape. Thus, knowledge how materials lose their mechanical properties after implantation seems to be essential. The tensile strength, elongation at break and Young's modulus of PTMC-PLA films as a function of *in vitro* degradation are presented

in Figures 4-6, respectively. Tensile strength of PTMC-PLA copolymer was 2.55 MPa, elongation at break was 91.28%, what proved that obtained polymers is very soft and flexible. The Young's modulus was 9.78 MPa.

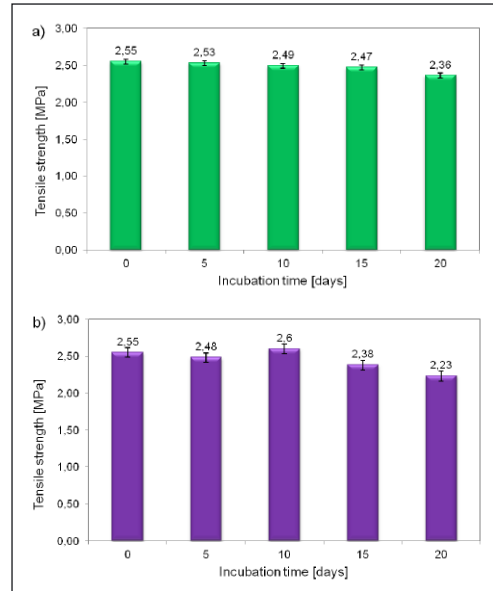


Figure 4. Tensile strength of PTMC-PLA films incubated in a) distilled water and b) Ringer's fluid

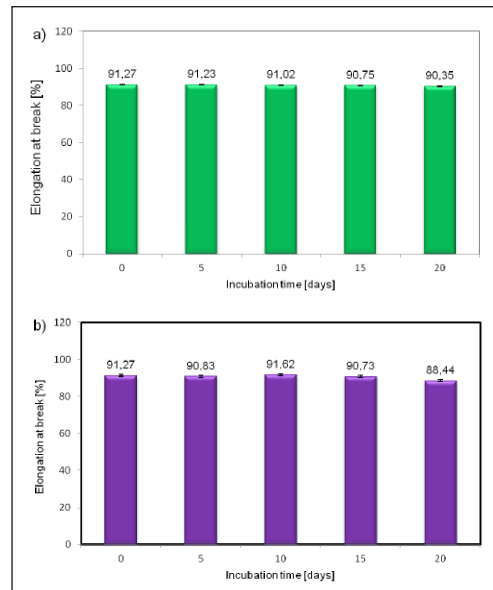


Figure 5. Elongation at break of PTMC-PLA films incubated in a) distilled water and b) Ringer's fluid

During incubation in distilled water tensile strength constantly decreased and after 20 days reached 2.36 MPa. However, the tensile strength of films incubated in Ringer's fluid initially decreased, then increased and decreased again till the end of measurement and was 2.23 MPa. This reinforcement effect during *in vitro* degradation may be caused by swelling of the PLA units [41]. The elongation at break in both liquids slightly decreased but still remains relatively high to maintain the shape of the implant without collapsing.

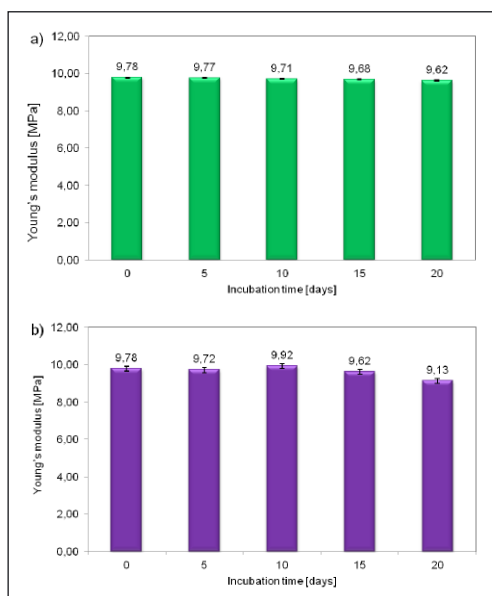


Figure 6. Young's modulus of PTMC-PLA films incubated in a) distilled water and b) Ringer's fluid

The Young's modulus of PTMC-PLA films incubated both in distilled water and Ringer's fluid decreased, however, terminal value was a little bit lower in Ringer's fluid. The loss of the values is directly connected with random scission of the ester backbone of PTMC-PLA leading to a noticeable reduction of mechanical properties, despite the low rate of degradation of PTMC-PLA copolymer.

Surface properties are dominating factor in affecting the interaction between neural scaffold and neural cells. The surface hydrophobicity is

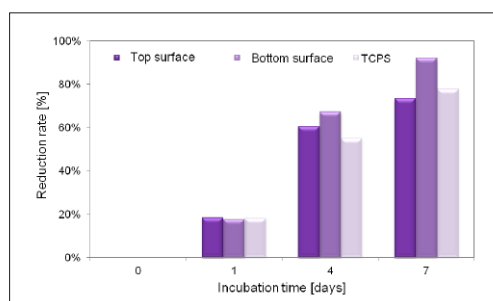
well known as a key factor to govern cell response. The lower the contact angle, the more hydrophilic the surface is. It is well known that both highly hydrophilic and highly hydrophobic surfaces are not favorable for cell attachment. Surfaces with moderate wettability are able to adsorb proper amounts of adhesive proteins and at the same time enable to preserve their natural conformations, what stimulates positive cell response [42] cellular and protein levels to biomaterials is, in most cases, closely associated with the materials' surface properties. In tissue engineering, regenerative medicine and many other biomedical fields, surface engineering of the bio-inert synthetic polymers is often required to introduce bioactive species that can promote cell adhesion, proliferation, viability and enhanced ECM-secretion functions. Up to present, a large number of surface engineering techniques for improving biocompatibility have been well established, the work of which generally contains three main steps: [1]. Material surface roughness (or topography) is another important factor influencing cell adhesion and behavior. Indeed, roughness modulates the biological response of tissues in contact with the implant. Material surface roughness has a direct influence *in vitro* as well as *in vivo* on cellular morphology and proliferation [43, 44]. The data from contact angle and profilometry measurements of PU/PLA blend are listed in Table 1. It can be concluded that PTMC-PLA film has smooth surface on both sides and no significant irregularities cannot be noticed. The upper surface of the polymer film is slightly less rough than the lower. The average level of roughness R_a of the top surface was 1.35 μm , whereas the bottom was 1.71 μm .

Examined surface of PTMC-PLA film has good surface wettability – the value of the contact angle below 90°. The top surface of the film is slightly more hydrophilic ($\theta = 68.3$) of the lower surface ($\theta = 70.1$). The Alamar Blue test was evaluate viability of fibroblasts on PTMC-PLA films, presented in Figure 7. One can notice that there are no sig-

Table 1. Water contact angle (θ) and roughness (R_a) of top and bottom surface of PTMC-PLA films

	θ [°]	R_a [μm]
TOP	68.3 ± 5.9	1.35 ± 0.23
BOTTOM	70.1 ± 4.1	1.71 ± 0.17

nificant differences after 24 h of culture and cell number is very similar for the top and the bottom surfaces of samples as well as for TCPS. It seems that in this case cell attachment and adhesion are independent on material surface properties. After 4 and 7 days, the cells number increased and was significant higher than on the control TCPS. However, the cell number on the top surface is slightly lower than on the bottom surface. It may indicate that fibroblasts adhere and proliferate more favourably on more hydrophilic and rougher surface.

**Figure 7.** Reduction rate of fibroblasts cells on top surface and bottom surface of PTMC-PLA films and TCPS as a control after 24 h, 4 and 7 days

Morphology and distribution of NIH 3T3 cells cultured on both surfaces (top and bottom) of the PTMC-PLA films as well as on the reference TCPS are presented in Figure 8. The fluorescence images showing live (green) and red (dead) cells correspond to the results of cell viability evaluated by Alamar Blue test.

On the bottom surface the single dead cell was observed after 24 h of culture. Adhered cells to the surface of PLLA-TMC showed normal morphology, but different from the morphology of the cells on TCPS. Fibroblasts on TCPS are well spread,

polygonal or spindle-shaped, what indicated that they were in resting state. While the majority of the cells on PTMC-PLA were irregular-shaped, round or oval, which confirmed that they were in active state. Moreover, part of the fibroblasts had a cubic shape, which showed that they were in the time of full activity.

Conclusions

The aim of this work was to examine how the degradation process affects the mechanical and surface properties of the copolymer of trimethylene carbonate and lactic acid in the light of biomedical applications. The copolymer undergoes hydrolytic degradation very slowly. Although, polylactides degrade via bulk erosion mechanism by the random scission of the ester backbone into lactic acid a normal human metabolic by-product, which is broken down into water and carbon dioxide via citric acid cycle. Enzymes role in the degradation process still is not fully explained. However, it is known that activity of enzymes is meaningless when the polymer is in its glassy state, but takes an important part when polymer is in elastic state. Thus, poly(trimethylene carbonate), which undergoes surface degradation with the rate of *in vivo* degradation was found to be much higher than *in vitro* degradation. This is presumably due to the contribution of *in vivo* enzymatic degradation process. The degradation *in vitro* test showed that the mass loss of samples incubated in simulated body fluid was higher than in distilled water. Therefore, Ringer's fluid turned out to be more aggressive environment for PTMC-PLA films. The pH changes in both media were mostly

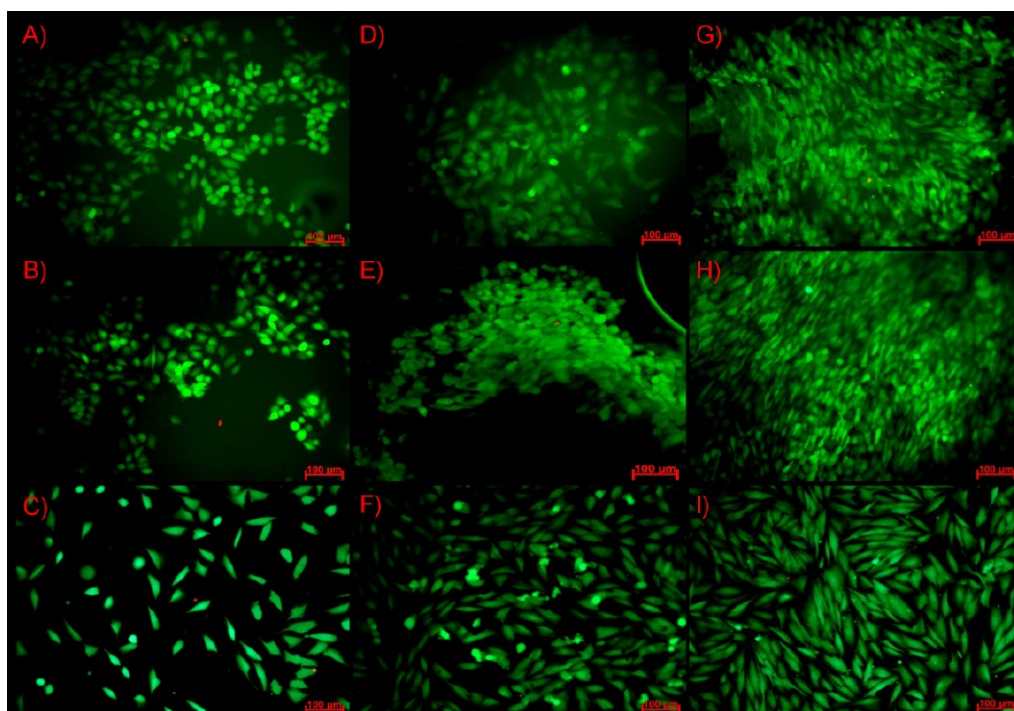


Figure 8. Morphology of NIH 3T3 cells cultured on the PTMC-PLA films: top surface (A, D, G), bottom surface (B, E, H) and reference samples TCPS (C, F, I) after 24 h (A ÷ C), after 4 days (D ÷ F) and 7 days (G ÷ I); fluorescence staining acridine orange, evaluation under fluorescence microscope, bar 100 μm

caused by steadily degradation of carbonate units into alkaline diols. The growth of ion conductivity value at the beginning of the incubation process was associated with the releasing of free ions to the solution. The mechanical parameters, namely, tensile strength, elongation at break and Young's modulus decreased with the progress of degradation process. The reduction of the mechanical properties resulted from the course of degradation and it was noticeable for samples incubated both in distilled water and in simulated biological environment. Ringer's fluid, as more aggressive, caused a higher decrease in mechanical properties. The measured contact angles showed good surface wettability. Both surfaces, the top and the bottom, have similar hydrophilicity. Moreover, activity of fibroblasts cells were similar on both sides as well as on the reference TCPS. Good adhesion of NIH 3T3 cells to the surface suggests that the

hydrophilic polymers promote colonization of fibroblasts cells on their surface. Biological studies have shown that used cells are very sensitive to surface topography which they colonize and cell viability was higher at the bottom surface, which has a slightly higher average roughness R_a . Thus, fibroblasts cell preferred colonizing rougher than smoother surfaces. Fabricated films does not affect negatively, namely, toxic on cell cultures and form substrate with favourable surface properties. This was confirmed by the Alamar Blue tests and microscopic observations.

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