In vitro evaluation of a multifunctional nano drug delivery system based on tigecycline-loaded calcium phosphate/poly-DL-lactide-co-glycolide

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Abstract

Most drug delivery systems as treatment modalities for osteomyelitis have not been evaluated for resistant infections. Tigecycline (TG) is an antimicrobial agent that could be used in the treatment of multi-drug-resistant orthopedic infections. The objective of this *in vitro* study has been to determine what dosage of TG causes changes in the morphology and number of osteoblasts. We have also investigated whether nanoparticulate tigecycline-loaded calcium-phosphate/poly(DL-lactide-co-glycolide) is biocompatible and whether it could release bioactive TG in a controlled manner during the observation time. The cytotoxicity was tested by analyzing the release of lactate dehydrogenase from dead osteoblasts to the medium. *Staphylococcus aureus* was used to verify the antibacterial effect of the multifunctional drug delivery system. At concentrations as achieved by local application, TG caused high toxic effect and impaired the normal osteoblastic morphology. The nanoparticulate multifunctional drug delivery system showed good compatibility and antibacterial effect during the observation time and thus appears to be suitable for the treatment of osteomyelitis caused by multi-drug-resistant microbes.

Keywords: calcium phosphate, poly-DL-lactide-co-glycolide, multifunctional, nano drug delivery

1. Introduction

The septic disease osteomyelitis represents the worst complication in orthopedic surgery and traumatology. It is difficult to ensure effective bone transplantation and to treat the infection at the same time. The systemic administration of antibiotics cannot provide sufficient local drug concentration and long-term administration may cause side-effects. In patients with bone defects, such treatments must have two stages, i.e. the control of the infection, as the first, and bone grafting as the second stage. In recent years, diverse antibiotic delivery systems have been developed as treatment modalities in infectious bone injuries or chronic osteomyelitis. Their advantage rests in the possibility to achieve a high local antibiotic concentration without exposing the patient to systemic toxic levels [1-6]. Most antibiotic delivery systems have not been evaluated for unusual or resistant infections and their impact remains limited in the rapidly changing field of orthopedic microorganisms. Finally, once drug elution has tapered off, the carrier becomes another foreign body that fosters bacterial colonization.

Multifunctional drug delivery systems (MDDS) are designed with the idea of ensuring a slow release of the drug, the elimination of the drug carrier and regeneration of the place at which the implant was placed. Poly(d,l-lactide-co-glycolide) (PLGA) is a biodegradable polymer with non-toxic degradation products that are easily removed from the organism by natural metabolite pathways. Nanoparticulate systems based on PLGA have also been the subject of research in the treatment of osteomyelitis. Calcium-phosphate (CP) is chemically similar to the mineral component of bones and it has the capacity to promote and stimulate the regeneration of bone tissue [7-10].

Multifunctional nano drug delivery systems (MNDDS) for the controlled drug delivery based on calcium phosphates and bioresorbable polymers are the subject of our recent research related to bone engineering [7, 8, 11, 12]. The basic idea behind this concept is to exhibit the activity of MNNDS through two successive steps. The first step includes the controlled release of an antibiotic accompanied with polymer resorption. In the second step, CP particles coated with the polymer, which remain in place, act as filler for potential damages in bone tissue [7]. Composite biomaterials based on calcium phosphates in nanoparticulate form (NPs) compared to micro or submicron particles may have several advantages in terms of improving the quality of the treatment [13-15]. Different groups of composite biomaterials based on hydroxyapatite nano particles were synthesized for the purpose of reconstruction of bone tissue [16, 17].

Having in mind that currently used antibiotic therapy options have limited effect on antibiotic-resistant bacteria, such as gentamicin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), there is an urgent need for new multifunctional antibiotic delivery systems to combat these resistant pathogens [18-21]. Tigecycline (TG) is the first in the new class of antimicrobial agents, the glycylcyclines, which are structurally derived from the tetracycline nucleus. The antimicrobial activity of TG is based on the inhibition of protein synthesis in bacteria, by binding them to the 30S ribosome subunit, blocking the bond to the tRNA, on the mRNA–ribosome complex [22-25]. TG appears to find potential application in the treatment of severe orthopedic infections. Several case reports of the treatment of osteomyelitis, caused by multi-drug-resistant microbes, with TG have been published so far [26-28]. TG has displayed good bone penetration and a broad antimicrobial spectrum, including all the pathogens found in nosocomial orthopedic infections including MRSA [21].

The purpose of the study presented in this paper has been to examine the effects of different concentrations of tigecycline on osteoblast cells and find out the appropriate concentration of tigecycline in the multifunctional nano drug delivery system consisting of tigecycline-loaded CP/PLGA that can maintain antibacterial properties during potential applications in a living tissue. Despite the increasing use of tigecycline and the reported high concentrations in bone tissue, there are no data available concerning the direct effects of tigecyline on bone cells. The objective of this *in vitro* study has been to determine what dosage of tigecycline causes changes in the morphology of osteoblasts and reduces the number of osteoblast cells. We have also examined whether MNDDS based on tigecycline-loaded CP/PLGA is compatible and whether it can release bioactive TG in a controlled manner within three weeks; after that period, *in vitro* research were carried out.

2. Materials and Methods

2.1 Preparations of composite biomaterial

Synthesis of a tigecycline-loaded nano composite biomaterial in which each calcium phosphate particle was coated with the bioresorbable poly-DL-lactide-co-glicolide polymer, was described early [7]. The calcium phosphate gel was added into completely dissolved polymer containing different ratios of Tigecycline (Tygacil/Tigecyclin, Wyeth Europe Ltd., Berkshire, SL6 OPH). The suspension was mixed at 18,000 rev/min, and then methanol was added. Afterwards, PVA (0.02% in water) was added into the

suspension (PLGA/PVA = 10/1) [7]. Tigecyclin (T) content in the composite was obtained after calculating the encapsulation efficiency (%), according to the methodology presented in literature [29]; the encapsulation efficiency in our experiments was 70 ± 3%. Nano-sized particles of CP/PLGA (without tigecycline), CP/PLGA loaded with 0.6wt% tigecycline (CP/PLGA/TG0.6) and 5wt% tigecycline (CP/PLGA/TG5.0) were synthesized. Microstructural characterization was done by atomic force microscopy (AFM; Thermo Microscopes, Autoprobe CP Research). The samples were sterilized in a ^{60}Co radiation facility, in air at room temperature, at a dose rate of 9 kGy/h, to absorbed doses of 25 kGy before use [7]. X-ray (XRD) analyses were made using a Bruker D8 advance diffractometer equipped with focusing Ge crystal primary monochromator that generates CuK radiation. The chemical composition was identified by infrared spectroscopy, performed on a Michelson interferometer with duplex mechanical bearings and a linear motor, resolution 32–0.5 cm^{-1}, spectral range DTGS 7.8–400 cm^{-1}, and an accuracy lower than 0.01 cm^{-1}.

2.2 Cell Culture

The mouse osteoblast-like cell line, MC3T3-E1 (DSMZ, ACC210, Braunschweig, Germany) was routinely cultured in alpha-MEM (PAA Laboratories Gmbh, Austria) supplemented with 10% fetal calf serum (PAA Laboratories Gmbh, Pasching, Austria), 100 U/ ml penicillin, and 0.1 mg/ ml streptomycin (PAA Laboratories Gmbh) at 37°C in humidified atmosphere of 95% air and 5% CO_2. The test samples were placed in 24-well cell culture plate and seeded with 2 x 10^5 cells in 0.5 ml medium.

2.3 Cytotoxicity

The cytotoxicity [30] was tested by analyzing the release of lactate dehydrogenase (LDH) into the cell culture medium. LDH was measured using the microplate based Cytotoxicity Detection Kit (LDH) (Boehringer, Mannheim, Germany). According to the manufacturer's protocol, 50 µl of the cell culture medium was used and absorbance was measured at 492 nm using a plate reader (ASYS Hitech GmbH, EXPERT 96, Austria). The total protein content was measured using the microplate-based Pierce® BCA Protein Assay Kit and the working reagent was prepared according to the manufacturer's protocol; an amount of 250 µl of the cell culture medium was used and the absorbance was measured at 550 nm using a plate reader (ASYS Hitech GmbH, EXPERT 96, Austria). The culture medium without cells was used as a zero absorbance

reference to calibrate the spectrophotometer. LDH activities were calculated in percentage of the LDH activity against the total cell death induced by 1% Triton X-100 treatments (control).

2.4 Tigecycline treatment

Tigecycline antibiotic powder (Wyeth, USA) was solubilized following the manufacturers' instructions to obtain six different concentrations (0, 0.5, 5, 50, 250, and 500 μg/ml). The attachment of cells to the bottom of the culture plates was achieved after 24 h. The cells were incubated for another 24, 48 or 72 h before the assessment of the cell cytotoxicity and morphology. The cell medium was changed after 24 hours. The number of cells was 300,000 or 300,000 per hole in 1 ml. The results were expressed as a ratio of released LDH to the amount of untreated control cells.

2.5 CP/PLGA tigecycline treatment

After 24 h, the attachment of cells was achieved, the medium was removed, and 1 ml of fresh medium was added to each well containing 0.02 g pulver of CP/PLGA (without tigecycline), CP/PLGA/TG0.6 and CP/PLGA/TG5 [21]. Cells were then incubated for another 24, 48, and 72 h before the assessment of the cell cytotoxicity. We attempted to describe through quantitative parameters the manner in which extracts from CP/PLGA, CP/PLGA/TG0.6 and CP/PLGA/TG5 induce the LDH activity in the extracellular space. The medium was changed after 24, 48 and 72h.

2.6 Morphology

The morphology of the cells cultivated with different concentrations of TG and of those from the control (without tigecycline) culture were examined after 24, 48 and 72 hours using a light microscope with a 10x and a 40x objective (Olympus 1x70, Hamburg, Germany).

2.7 Verification of the microbiological activity of tigecycline-loaded CP/PLGA

In order to test the antibacterial activity of the released TG from tigecycline-loaded CP/PLGA, the growth inhibition of the most common organism causing osteomyelitis, *S. aureus*, was used [20,31]. *Staphylococcus aureus* ATCC 10832 was inoculated on the surface of nutrient agar plates. Six sterile blank 6-mm paper discs were placed on the surface of each inoculated agar plate. Each disc was loaded with 50μl of the standard

TG solutions and incubated for 24 h, after which the diameters of the zones of complete inhibition (including the diameter of the disc) were recorded in millimeters. The concentrations were then plotted on a semilogarithmic scale versus their corresponding zone diameters to give a standard curve. Six-millimeters discs of CP/PLGA, CP/PLGA/TG0.6 and CP/PLGA/TG5 were incubated in sterile saline for 24 h, seven, 14 and 21 days and after that, they were applied in triplicate to the prepared bioassay agar plates incubated at 30°C for 24 hours. The diameters of the zones of inhibition for the standards and the samples were measured manually. The six measurements per zone of inhibition (three measurements per sample × two replicates) were averaged. The concentrations of the samples were determined by comparing the mean zone size of the samples to the zone sizes of the standard curve and their corresponding concentrations. All measurements were documented with photos and performed by a person who was not involved in the research.

2.8 Statistical analysis

The statistical analysis was performed using the software package Sigma Plot version 11.0. Groups of data were evaluated using a one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis tests for all pair wise multiple comparisons or Dunn's for multiple comparisons versus the control group. The significance was set at $p < 0.001$. The data were presented as the mean ± standard error. All experiments were done thrice with a minimum of four samples for each concentration and parameter.

3. Results

3.1 Tigecycline-loaded calcium phosphate/poly-DL-lactide-co-glycolide basic analysis

Difraktogram of tigecycline-loaded calcium phosphate/poly-DL-lactide-co-glycolide loaded with 5wt% tigecycline (specimens with maximum ratio of TG) is shown in Figure 1a. In Figure 1b shows the FT-IR spectrum of the same material.

The most intense peaks, in Figure 1a, at 31.8^0 (2 1 1), 32.2^0 (1 1 2), 32.9^0 (3 0 0) and 49.5^0 (2 1 3) originate from calcium hydroxyapatite (HAp). Peaks at 16.9^0, 18.2^0, 23.1^0 and 43.0^0 derived from tigecycline. The FT-IR spectra shown in Figure 1b are observed doublets with maxima at 1,035 and 1,092 cm^{-1}, which are the most intense and originate from phosphate groups, and by a triplet with maxima at somewhat lower frequencies of 561 and 603 cm^{-1}, arising from the $PO4^{3-}$ group vibrations [7]. The intense, sharp band

at 1,760 cm^{-1} is attributed to C=O vibration from PLGA. The band at 1,521 cm^{-1} corresponds to the most intense peaks from tigecycline $C_{29}H_{39}N_5O_8$.

The microstructure of CP/PLGA and CP/PLGA/TG composite biomaterial was studied by AFM. Fig. 2 shows the morphology of the the CP/PLGA, CP/PLGA/TG0.6 and CP/PLGA/TG5 composite biomaterial prepared according to experimental procedures. It was established by linear AFM analysis that the average particle diameter of this composite varied from 65 to 95 nm.

Figure 1.

Figure 2.

3.2 Cell morphology

A treatment with TG leads to considerable changes in the cell morphology and the cell spread on the surface. After 72 hours of incubation, the control osteoblast-like cells were uniformly distributed on the culture plate and they exhibited the characteristic cuboidal morphology (figure 3a). The cell borders were well defined; the cells were attached to the surface and exhibited a spread, flattened shape (figure 3a). We observed no changes in the cellular morphology when the osteoblasts were treated with 0.5 μg/mL, 5 μg/mL and 50 μg/mL TG (figure 3b). At a concentration of 250 μg /mL TG, there were fever cells in the focus and the cells became more fibroblast-like with a spindle shape and with long processes extending between cells (figure 3c). At 500 μg /mL, this tendency was even more pronounced (figure 3d).

Figure 3.

3.3 Lactate dehydrogenase assay

All doses of TG greater than 50μg/mL on days 1, 2 and 3 resulted in an increased ($p < 0.001$) release of LDH compared to that of the control cells (figure 4). Although the data collected on days 1 and 2 showed a similar downward trend when the osteoblasts were treated with 0.5 μg/mL, 5 μg/mL TG, there have been no significant differences from the controls. As far as the time effect at each concentration of TG is concerned, the LDH activity did not significantly change at any concentration of the antibiotic during the observation period.

Compared to the corresponding controls at each time point, CP/PLGA/TG0.6 did not reveal cytotoxic effects (figure 5). A significantly decreased LDH activity was observed in the CP/PLGA/TG5 incubated for between 24 and 72 h ($p < 0.001$).

Figure 4.

Figure 5.

3.4 Microbiological activity of the released tigecycline from tigecycline-loaded CP/PLGA

The results of the TG biological activity test are presented as the mean value in table 1. CP/PLGA did not exhibit any biological activity. CP/PLGA/TG0.6 and CP/PLGA/TG5 showed an average zone of inhibition of 19 and 30 mm, respectively. The zones of inhibition indicate that biologically active TG is present in the sample. Different biological effect of the samples corresponds to different concentrations of the antibiotic. The inhibitory activity of the diluents and their effect on the standard curve did not allow for the quantification of the amount of TG.

Table 1.

4. Discussion

XRD analysis results indicate the presence of calcium phosphate phases and tigecycline in the synthesized powder. Form of XRD peaks in Figure 1a shows the existence of poorly crystalline calcium hydroxyapatite. PLGA polymer because of its amorphous structure does not show the diffraction peaks. Based on the position and intensity of the characteristic peaks of tigecycline (16.9°, 18.2°, 23.1° and 43.0°) it is likely that the given phase is tigecycline crystalline form I. There are different crystalline forms of TG and the results indicate the existence of consent forms I [32, 33].

FTIR spectrum is shown in Figure 1b confirmed the existence of HAp, TG and PLGA phase in the synthesized powder.

The AFM image of the composite without tigecycline is shown in Fig. 2a and it indicates an average particle diameter of around 65 nm. The AFM morphology of the composite containing 0.6% and 5% of tigecycline is shown on Fig. 2b, c. Undoubtedly, the increase in the tigecycline content within the composite leads to the increased average particle diameter up to 95nm. In accordance with the XRD and FTIR results, CP particles coated with tigecycline-loaded PLGA were obtained. In our previous studies it was shown that the average particle size ranges of the nano-sized powder between 65 and 95 nm [7].

Although the efficacy of the local antibiotic delivery systems is beyond dispute, the effect of high doses of antibiotics on local tissues is not well covered in relevant literature. The literature data indicate that increased antibiotic concentrations are accompanied with an increase in toxicity, suggesting that higher doses of the antibiotic may be better for the control of the infection; however, they are by no means benign [34]. It is important to take into consideration that most local delivery systems release a very high dose of antibiotics; in some cases the concentration is higher than 1000 times the MIC 90 [35-38].

To our knowledge, the present study has demonstrated the inhibitory effects of pure TG and the TG released from tigecycline-loaded CP/PLGA on osteoblasts *in vitro* for the first time. In the interpretation of our data, we have noted important caveats. First, we focused on the cytotoxicity and did not evaluate the cellular function in detail. The full bio programme has been beyond the scope of this paper. In addition, we evaluated the effect of the antibiotics in an *in vitro system* and our findings may not be directly applicable to an *in vivo* situation.

We have shown that the TG treatment at serum and bone concentrations achieved in a conventional intravenous antibiotic therapy 0.5 μg/ml – 5 μg/ml neither yields toxic effects nor causes morphological change in osteoblasts *in vitro* [31,37]. A concentration of tigecycline one-hundred-fold higher than MIC 50 for *coagulase-negative staphylococci* (CNS) did not cause negative effects on osteoblasts, which indicates that tigecycline could be used as a part of MDDS [39]. Higher concentrations, 250 μg/ml and above, caused high toxic effect and impaired normal osteoblastic morphology, indicating a fibroblastic rather than osteoblastic phenotype, maybe in a sense of dedifferentiation. The effect of the concentrations of TG, 500- or 1000-fold higher than MIC 50 for CNS, indicates that a MDDS based on TG could be compatible only if it had a well controlled antibiotic release rate. The cytotoxic and cytostatic effects of tetracycline on osteoblasts could be explained by a mitochondrial energetic impairment. Tetracycline is known to inhibit the bacterial protein synthesis, as well as the eukaryotic protein synthesis and protein synthesis of isolated mitochondria [37, 40-42]. Significant morphological changes of cells in spread, cell membrane, and extensions were also detected with the use of other antibiotics (ciprofloxacin, vancomycin) over the dose 100 μg/ml [43].

We have demonstrated that there was no impact on the percentage of viable osteoblasts incubated with CP/PLGA in the first 24 h, which is in accordance with our earlier

research [44]. Low cytotoxicity values are probably the consequence of high compatibility of both constituents of the biocomposite material. The investigation of the time effect on each sample showed that the extracts from CP/PLGA increased significantly the LDH activity at each time point, but this activity was no more than two folds higher than that of the controls. Faster degrading polymers, like low–molecular-weight PLGA or PLA, can give rise to a cytotoxic reaction due to the production of acidic degradation products [45-47].

Compared to the corresponding controls at each time point, CP/PLGA/TG0.6 did not reveal cytotoxic effects. During the first day, eight percent of the total TG was released, calculated 9.6 μg of TG; [7] this concentration of TG did not cause cytotoxic effects. The lower LDH activity in the cells incubated with CP/PLGA/TG0.6 than that in the cells incubated with CP/PLGA can be explained by a positive proliferation effect of tetracycline on osteoblasts [40]. The mechanisms underlying the effects of tetracyclines on osteoblastic cells remain unclear. However, tetracyclines can inhibit collagenase and/or the breakdown of collagen under a variety of conditions. These events might result in improved osteoblastic behaviour, having in mind that a stable collagenous matrix is known to play a significant role in the osteoblastic proliferation/differentiation sequence [48-50]. CP/PLGA/TG5 significantly decreased the LDH activity from 24 to 72 h. This can be explained by the high TG release in the first day and its deleterious effect on osteoblasts. During the first day, 20% of the total TG was released – namely, 200μg of TG. The highest release rate during the first day is related to the fact that a great amount of the antibiotic is adsorbed on the surface of nanoparticles, which is the consequence of the synthesis procedure. The zones of inhibition around the samples indicate that biologically active TG remains in the sample for three weeks. The quantification and the release mechanism of TG from implants *in vivo* and *in vitro*, important for potential preclinical applications, were dealt with in our previous study [7].

5. Conclusions

Multifunctional nano drug delivery systems (MNDDS) based on tigecycline-loaded calcium phosphate/poly-DL-lactide-co-glicolide have shown good compatibility and antibacterial effect during the observation time and thus appear to be a suitable drug delivery system for the treatment of osteomyelitis caused by multidrug-resistant

microbes. Tigecycline-loaded calcium phosphate/poly-DL-lactide-co-glycolide with 0.6wt% of tigecycline shows a low level of the cytotoxic and LDH activity.

This study provides additional rationale for an improved patient dosing and the necessity for a balance between the antibacterial and biocompatible properties of multifunctional local delivery systems. Tigecycline treatment at concentrations achieved in a conventional intravenous antibiotic therapy did not yield toxic effects or caused morphological changes of osteoblasts *in vitro*. However, at high concentrations as achieved by local application, tigecycline caused high toxic effect and impaired normal osteoblastic morphology; therefore it may be detrimental for bone healing and repair *in vivo*.

Further *in vitro* studies should highlight the effects of tigecycline on the metabolic activity, extracellular matrix calcification and differentiation of human osteoblasts. Additional *in vivo* studies should focus on a prolonged effect of high tigecycline levels on bone healing and implant incorporation.

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REFERENCES

[1] Bi L, Hu Y, Fan H, Meng G, Liu J, Li D, Lv R. Treatment of contaminated bone defects with clindamycin-reconstituted bone xenograft-composites. J. Biomed. Mater. Res. Part B Appl. Biomater. 2007;82B:418-27. [http://dx.doi.org/10.1002/jbm.b.30747]

[2] Cornell C, Tyndall D., Waller S, Lane J, Brause B. Treatment of experimental osteomyelitis with antibiotic impregnated bone graft substitute. J. Orthop. Res. 1993;11:619–626. [http://dx.doi.org/10.1002/jor.1100110502]

[3] Duewelhenke N, Krut O, Eysel P. Influence on mitochondria and cytotoxicity of different antibiotics administered in high concentrations on primary human

osteoblasts and cell lines. Antimicrob. Agents Chemother. 2007;51:54-63. [http://dx.doi.org/10.1128/AAC.00729-05]

[4] Korkusuz F, Korkusuz P, Eksioglu F, Gursel I, Hasirci V. In vivo response to biodegradable controlled antibiotic release systems, J. Biomed. Mater. Res. 2001;55:217–28. [http://dx.doi.org/10.1002/1097-4636(200105)55:2<217::AID-JBM1008>3.0.CO;2-Y]

[5] Zalavras C, Patzakis M, Holtom P. Local antibiotic therapy in the treatment of open fractures and osteomyelitis. Clin. Orthop. Relat. Res. 2004;427:86–93. [http://dx.doi.org/10.1097/01.blo.0000143571.18892.8d]

[6] Antoci V, Adams C, Hickok N, Shapiro I, Javad P. Antibiotics for local delivery systems cause skeletal cell toxicity in vitro. Clin. Orthop. Relat. Res. 2007;462:200–206. [http://dx.doi.org/10.1097/BLO.0b013e31811ff866]

[7] Ignjatović N, Ninkov P, Sabetrasekh R, Uskoković D. A novel nano drug delivery system based on tigecycline-loaded calcium phosphate coated with poly-DL-lactide-co-glycolide. J. Mater. Sci. - Mater. Med. 2010;21:231–239. [http://dx.doi.org/10.1007/s10856-009-3854-6]

[8] Vukomanović M, Škapin S, Jančar B, Maksin T, Ignjatović N, Uskoković V, Uskoković D. Poly(d,l-lactide-co-glycolide)/hydroxyapatite core-shell nanospheres. Part 1: A multifunctional system for controlled drug delivery. Colloids Surf. B 2011;82:404–13. [http://dx.doi.org/10.1016/j.colsurfb.2010.09.011]

[9] Zhongli S, Xin H, Bing L, Huimin T, Yurong C, Ruikang T. Biological response of osteosarcoma cells to size-controlled nanostructured hydroxyapatite. J. Biomater. Appl. 2010;25:19-37. [http://dx.doi.org/10.1177/0885328209339396]

[10] Najman S, Đordević Lj, Savić V, Ignjatović N, Uskoković D. Biological evaluation of hydroxyapatite/poly-l-lactide composite biomaterials with poly-l-lactide of different molecular weights intraperitonealy implanted into mice. Biomed. Mater. Eng. 2004;14:61-70.

[11] Ignjatović N, Liu C, Czernuszka J, Uskoković D. Micro and nano/injectable composite biomaterials of calcium phosphate coated with poly(dl-lactide-co-glycolide). Acta Biomater. 2007;3:927-935. [http://dx.doi.org/10.1016/j.actbio.2007.04.001]

[12] Vukomanović M, Škapin S, Poljanšek I, Žagar E, Kralj B, Ignjatović N, Uskoković D. Poly(D,L-lactide-co-glycolide)/hydroxyapatite core–shell nanosphere. Part 2: Simultaneous release of a drug and a prodrug (clindamycin and clindamycin

phosphate). Colloids Surf. B 2011;82:414–421. [http://dx.doi.org/10.1016/j.colsurfb.2010.09.012]

[13] Nelson M, Balasundaram G, Webster T. Increased osteoblast adhesion on nanoparticulate crystalline hydroxyapatite functionalized with KRSR. Int. J. Nanomedicine. 2006;1:339-349.

[14] Kumari A, Yadav S, Yadav S. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf. B 2010;75:1-18. [http://dx.doi.org/10.1016/j.colsurfb.2009.09.001]

[15] Ignjatović N, Ajduković Z, Savić V, Uskoković D. Size effect of calcium phosphate coated with poly-DL-lactide-co-glycolide on healing processes in bone reconstruction. J. Biomed. Mater. Res. Part B Appl. Biomater. 2010;94:108-117. [DOI: 10.1002/jbm.b.31630]

[16] Li Q, Wu M, Tang L, Zhou J, Jiang Y, Darvell B. Bioactivity of a novel nano-composite of hydroxyapatite and chitosan-phosphorylated chitosan polyelectrolyte complex. J. Bioact. Compat. Polym., 2008;23:520-531. [http://dx.doi.org/10.1177/0883911508097370]

[17] Ma X, Wang Y, Guo H, Wang J. Nano-hydroxyapatite/chitosan sponge-like biocomposite for repairing of rat calvarial critical-sized bone defect. J. Bioact. Compat. Polym. 2011;26:335-346. [http://dx.doi.org/10.1177/0883911511407402]

[18] Huang V, Cheung C, Kaatz G, Rybak M. Evaluation of dalbavancin, tigecycline, minocycline, tetracycline, teicoplanin and vancomycin against community-associated and multidrug-resistant hospital-associated meticillin-resistant Staphylococcus aureus. Int. J. Antimicrob. Agents 2010;35:25-29. [http://dx.doi.org/10.1016/j.ijantimicag.2009.08.020]

[19] Putnam S, Sader H, Farrell D, Jones R. Sustained antimicrobial activity of tigecycline against methicillin-resistant Staphylococcus aureus (MRSA) from United States Medical Centers from 2004 through 2008. J. Chemother. 2010;22:13-16.

[20] Calhoun J, Manring M, Shirtliff M. Osteomyelitis of the long bones, Semin. Plast. Surg. 2009;23:59-72. [http://dx.doi.org/10.1055/s-0029-1214158]

[21] Yin L, Lazzarini L, Li F, Stevens M, Calhoun, J. Comparative evaluation of tigecycline and vancomycin, with and without rifampicin, in the treatment of methicillin resistant Staphylococcus aureus experimental osteomyelitis in a rabbit

model. J. Antimicrob. Chemother. 2005;55:995–1002. [http://dx.doi.org/10.1093/jac/dki109]

[22] Manfredi R, Sabbatani S. Novel pharmaceutical molecules against emerging resistant gram-positive cocci. Braz. J Infect. Dis. 2010;14:96-108. [http://dx.doi.org/10.1590/S1413-86702010000100020]

[23] Bouchillon S, Hoban D, Johnson B, Johnson J, Hsiung A, Dowzicky M. In vitro activity of tigecycline against 3989 gram-negative and gram-positive clinical isolates from the United States tigecycline evaluation and surveillance trial (T.E.S.T. Program; 2004). Diagn. Microbiol. Infect. Dis. 2005;52:173–179. [http://dx.doi.org/10.1016/j.diagmicrobio.2005.06.004]

[24] Fritsche T, Sader H, Stilwell M, Dowzicky M, Jones R. Potency and spectrum of tigecycline tested against an international collection of bacterial pathogens associated with skin and soft tissue infections (2000–2004). Diagn. Microbiol. Infect. Dis. 2005;52:195–201. [http://dx.doi.org/10.1016/j.diagmicrobio.2005.05.003]

[25] Hoban D, Bouchillon S, Johnson B, Johnson J, Dowzicky M. In vitro activity of tigecycline against 6792 gram-negative and gram-positive clinical isolates from the global tigecycline evaluation and surveillance trial (T.E.S.T. Program, 2004). Diagn. Microbiol. Infect. Dis. 2005;52:215–227. [http://dx.doi.org/10.1016/j.diagmicrobio.2005.06.001]

[26] Schafer J, Mangino J. Multidrug-resistant acinetobacter baumannii osteomyelitis from Iraq. Emerg. Infect. Dis. 2008;14:512-14.

[27] Stanzani M, Tumietto F, Giannini M, Bianchi G, Nanetti A, Vianelli N, Arpinati M, Giovannini M, Bonifazi F, Bandini G, Baccarani M. Successful treatment of multi-resistant pseudomonas aeruginosa osteomyelitis after allogeneic bone marrow transplantation with a combination of colistin and tigecycline. J. Med. Microbiol. 2007;56:1692-695. [http://dx.doi.org/10.1099/jmm.0.47286-0]

[28] Kandemir O, Oztuna V, Colak M, Akdag A, Camdeviren, H. Comparison of the efficacy of tigecycline and teicoplanin in an experimental methicillin-resistant Staphylococcus aureus osteomyelitis model. J Chemother. 2008;20:53-57.

[29] Stevanović M, Savić J, Jordović B, Uskoković D. Fabrication, in vitro degradation and the release behaviours of poly(DL-lactide-co-glycolide) nanospheres containing ascorbic acid. Colloids Surf B Biointerfaces. 2007;59:215–223. [http://dx.doi.org/10.1016/j.colsurfb.2007.05.011]

[30] Decker T, Lohmann-Matthes M. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods. 1988;115:61-69. [http://dx.doi.org/10.1016/0022-1759(88)90310-9]

[31] Ji A, Saunders P, Wadgaonkar N, Petersen P, O'Leary K, McWilliams W, Amorusi P, Leal M, Fluhler, E. A novel antibiotic bone assay by liquid chromatography/tandem mass spectrometry for quantitation of tigecycline inrat bone. J. Pharm. Biomed. Anal. 2007;44:970–79. [http://dx.doi.org/10.1016/j.jpba.2007.04.017]

[32] Krishnan L, Deshmukh S, Hadfield A, Huang W, Ku M. Crystalline solid forms of tigecycline and methods of preparing same. US20070123497 A1 (Patent) 2007.

[33] Tsiperman E, Levi S, Aronhime J, Koltai T. Tigecycline crystalline forms and processes for preparation thereof. US20080090789 A1 (Patent) 2008.

[34] Huddleston P, Steckelberg J, Hanssen A, Rouse M, Bolander M, Patel R. Ciprofloxacin inhibition of experimental fracture healing. J. Bone Joint. Surg. Am. 2000;82:161–73.

[35] McLaren A, McLaren S, Nelson C, Wassell D, Olsen K. The effect of sampling method on the elution of tobramycin from calcium sulfate. Clin. Orthop. Relat. Res. 2002;403:54–57. [http://dx.doi.org/10.1097/00003086-200210000-00010]

[36] McLaren A. Alternative materials to acrylic bone cement for delivery of depot antibiotics in orthopaedic infections. Clin. Orthop. Relat. Res. 2004;427:101–106. [http://dx.doi.org/10.1097/01.blo.0000143554.56897.26]

[37] Mullangi P, Pankey G. Tigecycline in critical care. Crit. Care Clin. 2008;24:365-75. [http://dx.doi.org/10.1016/j.ccc.2007.12.007]

[38] Stevens M, Tetsworth K, Calhoun J, Mader J. An articulated antibiotic spacer used for infected total knee arthroplasty: a comparative in vitro elution study of simplex and palacos bone cements. J. Orthop. Res. 2005;23:27–33. [http://dx.doi.org/10.1016/j.orthres.2004.03.003]

[39] Conte J, Golden J, Kelly M, Zurlinden E. Steady-state serum and intrapulmonary pharmacokinetics and pharmacodynamics of tigecycline. Int. J. Antimicrob. Agents. 2005;25:523–29. [http://dx.doi.org/10.1016/j.ijantimicag.2005.02.013]

[40] Gomes P, Fernandes M. Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow

osteoblastic cells. Arch. Oral. Biol. 2007;52:251-59. [http://dx.doi.org/10.1016/j.archoralbio.2006.10.005]

[41] Rubinstein E, Vaughan D. Tigecycline: A novel glycylcyline. Drugs 2005;65:1317-336. [http://dx.doi.org/10.2165/00003495-200565100-00002]

[42] Verkade E, Verhulst C, Huijsdens X, Kluytmans J. In vitro activity of tigecycline against methicillin-resistant Staphylococcus aureus, including livestock-associated strains. Eur. J. Clin. Microbiol. Infect. Dis. 2010;29:503-507. [http://dx.doi.org/10.1007/s10096-010-0886-2]

[43] Antoci V Jr, Adams C, Hickok N, Shapiro I, Parvizi J. Antibiotics for local delivery systems cause skeletal cell toxicity in vitro. Clin. Orthop. Relat. Res. 2007;462:200-6. [http://dx.doi.org/10.1097/BLO.0b013e31811ff866]

[44] Ignjatović N, Ninkov P, Kojić V, Bokurov M, Srdić V, Krnojelac D, Selaković S, Uskoković D. Cytotoxicity and fibroblast properties during in vitro test of biphasic calcium phosphate/poly-dl-lactide-co-glycolide biocomposites and different phosphate materials. Microsc. Res. Tech. 2006;69:976-82. [http://dx.doi.org/10.1002/jemt.20374]

[45] Bostman O, Hirvensalo E, Vainionpaa S, Makela A, Vihtonen K, Tormala P, Rokkanen P. Ankle fractures treated using biodegradable internal-fixation. Clin. Orthop. Relat. Res. 1989;238:195–203.

[46] Fu K, Pack D, Klibanov A, Langer R. Visual evidence of acidic environment within degrading poly (lactic-co-glycolic acid) (PLGA) microspheres. Pharm. Res. 2000;17:100–06. [http://dx.doi.org/10.1023/A:1007582911958]

[47] Ignatius A, Betz O, Augat P, Claes L. In vivo investigation on composites made of resorbable ceramics and poly(lactide) used as bone graft substitutes. J. Biomed. Mater. Res. 2001;58:701–09. [http://dx.doi.org/10.1002/jbm.10024]

[48] Salasznyk R, Williams W, Boskey A, Batorsky A, Plopper G. Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. J. Biomed. Biotechnol. 2004;1:24–34. [http://dx.doi.org/10.1155/S1110724304306017]

[49] Gerstenfeld L, Chipman S, Kelly C, Hodgens K, Lee D, Landis W. Collagen expression, ultrastructural assembly, and mineralization in cultures of chicken embryo osteoblasts. J. Cell. Biol. 1988;106:979–89. [http://dx.doi.org/10.1083/jcb.106.3.979]

[50] Gomes P, Santos J, Fernandes M. Cell-induced response by tetracyclines on human bone marrow colonized hydroxyapatite and bonelike. Acta Biomater. 2008;4:630-37. [http://dx.doi.org/10.1016/j.actbio.2007.12.006]

Figure and table caption

Figure 1. Tigecycline-loaded calcium phosphate/poly-DL-lactide-co-glycolide (a) XRD diffraction (b) FT-IR spectra.

Figure 2. AFM image of **(a)** CP/PLGA), (b) CP/PLGA/T0.6, (c) CP/PLGA/T5.0.

Figure 3. MC3T3-E1 osteoblast-like cell cultures incubated with (a) 0, (b) 50 μg/mL, (c) 250 μg/mL, (d) 500 μg/mL TG in alpha-MEM medium for 72 hours, visualized by light microscope with original magnification ×40.

Figure 4. Specific LDH activity after one, two and three days in the medium with cell line MC3T3 incubated with different concentrations of TG 0-500 μg/mL.

Figure 5. Specific LDH activity after one, two and three days in the medium with cell line MC3T3 incubated with CP/PLGA, CP/PLGA/TG0.6 and CP/PLGA/TG5.0.

Table 1. The zone of inhibition around CP/PLGA, CP/PLGA/TG0.6 and CP/PLGA/TG5.0 determined by the microbiological assay using *S. aureus.* The values are presented as the mean value of a triplicate.

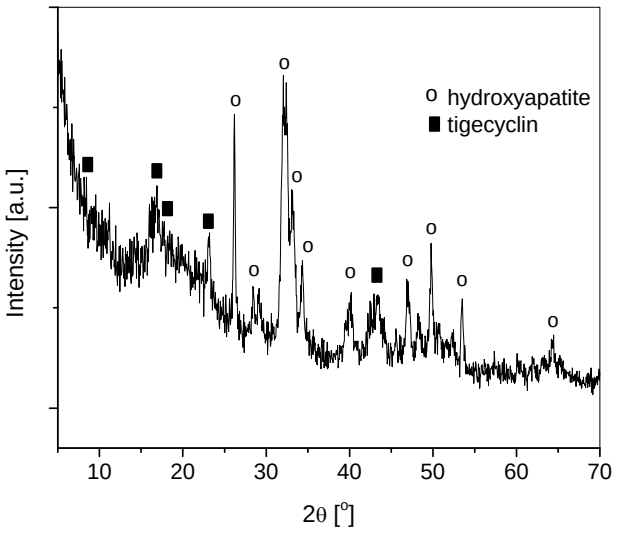


Figure 1a.

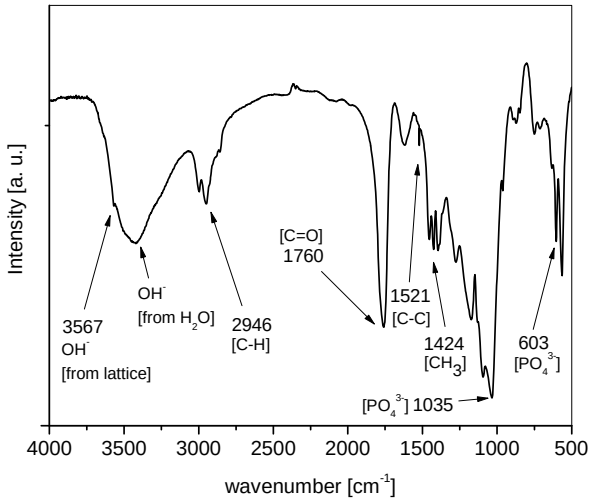


Figure 1b.

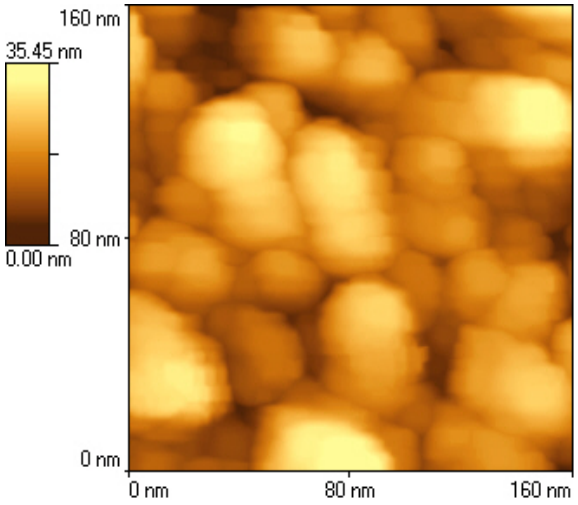
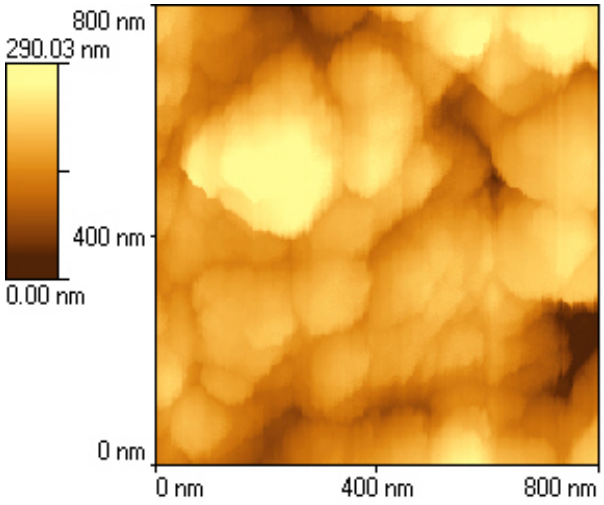


Figure 2a.



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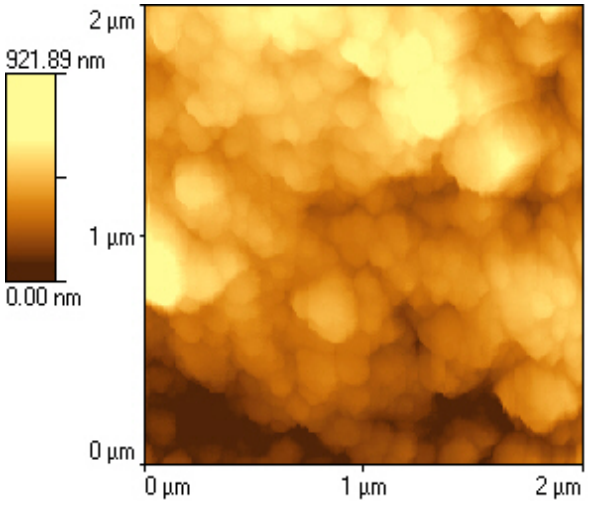


Figure 2c

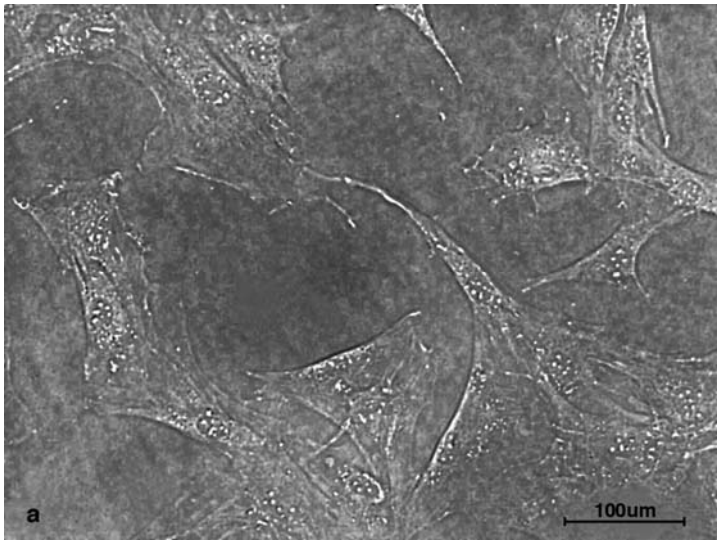
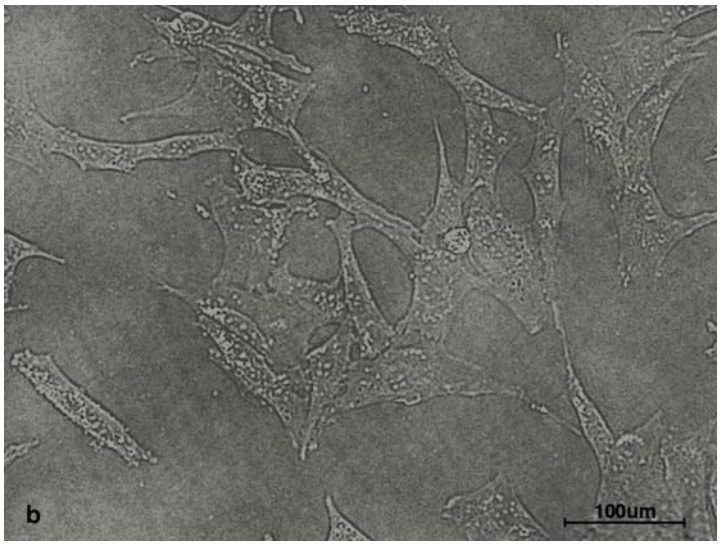


Figure 3a.



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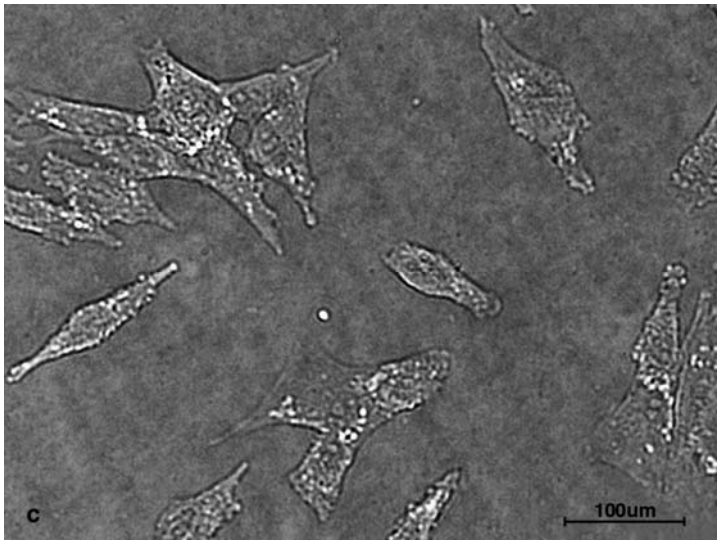

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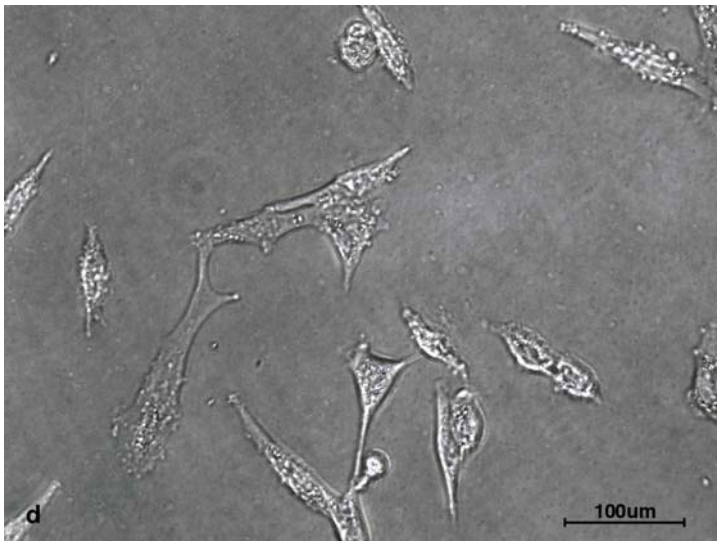

Figure 3d.

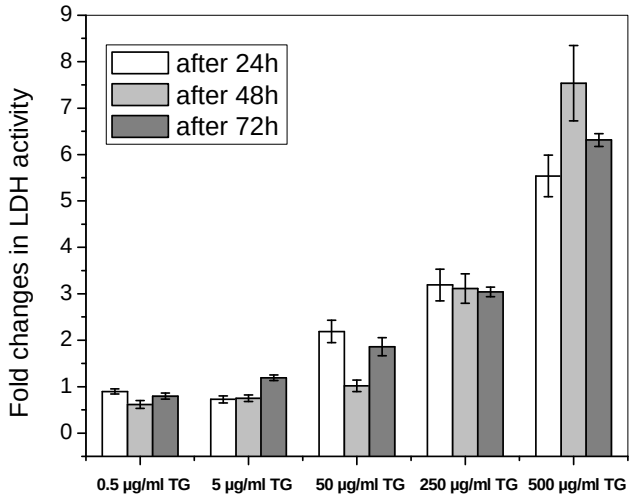

Figure 4.

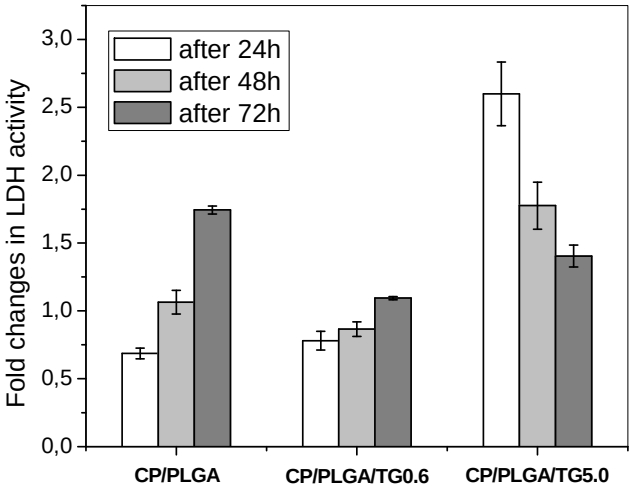

Figure 5.

Table 1.

incubation time [days]	inhibition zone [mm]		
	CP/PLGA	**CP/PLGA/T0.6**	**CP/PLGA/T5.0**
1	0	19	30
7	0	21	30
14	0	20	25
21	0	19	22