J Mater Sci: Mater Med (2010) 21:231–239
DOI 10.1007/s10856-009-3854-6

A novel nano drug delivery system based on tigecycline-loaded calciumphosphate coated with poly-DL-lactide-co-glycolide

Nenad L. Ignjatović · Petar Ninkov · Roya Sabetrasekh · Dragan P. Uskoković

Received: 9 February 2009 / Accepted: 13 August 2009 / Published online: 26 August 2009

Abstract The purpose of the study presented in this paper has been to examine the possibility of the synthesis of a new nanoparticulate system for controlled and systemic drug delivery with double effect. In the first step, a drug is released from bioresorbable polymer; in the second stage, after resorption of the polymer, non-bioresorbable calcium phosphate remains the chief part of the particle and takes the role of a filler, filling a bone defect. The obtained tigecycline-loaded calcium-phosphate(CP)/poly(DL-lactide-co-glycolide)(PLGA) nanoparticles contain calcium phosphate coated with bioresorbable polymer. The composite was analyzed by FT-IR, XRD and AFM methods. The average particle size of the nanocomposite ranges between 65 and 95 nm. Release profiles of tigecycline were obtained by UV–VIS spectroscopy in physiological solution at 37°C. Experimental results were analyzed using Peppas and Weibull mathematical models. Based on kinetic parameters, tigecycline release was defined as non-Fickian transport. The cytotoxicity of the nanocomposite was examined on standard cell lines of MC3T3-E1, in vitro. The obtained low values of lactate dehydrogenase (LDH) activity (under 37%) indicate low cytotoxicity level. The behaviour of the composite under real-life conditions was analyzed through implantation of the nanocomposite into living organisms, in vivo. The system with the lowest tigecycline content proved to be an adequate system for local and controlled release. Having in mind the registered antibiotics concentration in other tissues, delivery systems with a higher tigecycline content show both local and systemic effects.

N. L. Ignjatović · D. P. Uskoković (✉)
Institute of Technical Sciences of the Serbian Academy of Sciences and Arts, Belgrade, Serbia
e-mail: dragan.uskokovic@itn.sanu.ac.rs

P. Ninkov · R. Sabetrasekh
Faculty of Dentistry, Department of Biomaterials, University of Oslo, Oslo, Norway

1 Introduction

Several implantable antibiotics delivery systems have been developed for the treatment of bone infections. Their main disadvantage lies in the fact that implants should be removed at the end of the treatment. The advantages of localized biodegradable therapy for bone infections include high local antibiotics concentration at the site of infection. In order to achieve this, biodegradable implants of poly-caprolactone, with dispersed gatifloxacin antibiotic, were synthesized [1]. Several authors have also demonstrated the presence of gentamicin-resistant staphylococci after gentamicin bead therapy and reduced susceptibility or resistance to vancomycin, raising concern about the efficacy of this treatment option [2, 3]. Calcium phosphate bone cements containing tetracycline are used to fill bone defects and to ensure local antibiotherapy [4, 5]. As current antibiotic therapy options are becoming limited for staphylococcal infections, there is an urgent need for new antimicrobial agents to combat these resistant pathogens. Tigecycline is the first of a new class of antimicrobial agents, the glycylcyclines, which are structurally derived from the tetracycline nucleus. Tigecycline possesses activity against gram-positive and -negative pathogens, binding to the 30S ribosomal subunit and inhibiting protein synthesis [6, 7].

Bioresorptive polymers such as PLGA enable adhesion of cells, production of extracellular matrix and organization of cytoskeleton [8]. Coating of calcium-phosphate (CP) particles with PLGA polymer provides adhesion of osteoprogenitor cells with the final aim of intensifying differentiation and osteogenesis [9]. Calcium-phosphate/

poly(DL-lactide-co-glycolide) (CP/PLGA) composite biomaterial in granular form showed a high potential in the reconstruction of bone tissue damaged by osteoporosis [10, 11]. Low cytotoxicity justifies an increasing application potential of this kind of composite in human bone reconstruction [12]. It has been shown that a mixture of poly-lactide and poly-glycole copolymers, calcium phosphate ceramics (Osteosynt) and tetracycline antibiotic, leads to the reduction of inflammatory processes in human tissue [13]. Compared to pure polymers, the combination of CP with biodegradable polymers used in bone drug delivery systems shows certain advantages. PLGA micro spheres, with encapsulated gentamicin mixed with calcium phosphate bone cement, enable optimal use of this material in controlled release systems in bone engineering [14]. Poly-ε-caprolactone composite with dispersed gatifloxacine mixed with tri-calcium-phosphate can be used in the treatment of osteomyelitis [15]. PLGA micro spheres with encapsulated amoxicillin coated with HAp have potential applications in local bone delivery systems [16]. Composite biomaterials in nano particulate (NPs) form may have significant advantages over those in micro- or sub-micro-particulate form. Nano particles smaller than 100 nm have a larger specific surface area than microparticles, because the total surface area is inversely proportional to the third power of the diameter [17, 18].

The purpose of the study presented in this paper has been to examine the possibility of the synthesis of a two-step nanoparticulate system for controlled drug delivery; the system consists of calcium-phosphate (CP) nanoparticles coated with a layer of PLGA and operates in two successive steps. The first step includes controlled release of an antibiotic accompanied with polymer resorption. In the second step, CP particles coated with polymer, which remain, act as a filler for potential damages in bone tissue. To our knowledge, this is the first research concerning the production of nano spheres of tigecycline-loaded composite where calcium-phosphate is coated with poly(DL-lactide-co-glycolide) (PLGA) bioresorbable polymer. We analyzed possible tigecycline release mechanisms under static conditions by fitting the experimental data with the Peppas [19] and Weibull equations [20]. Possibilities for real-life application of this nanoparticulate composite biomaterial were tested under in vitro and in vivo conditions, by implantation into rats.

2 Materials and methods

2.1 Preparation of composite biomaterials

A calcium phosphate gel was prepared by precipitation of calcium nitrate and ammonium phosphate in an alkaline medium [10]. Poly(DL-lactide-co-glycolide) (PLGA) (50:50) (Sigma Chemical Company, USA) was used as a polymer component. Hydrolyzation degree of polyvinyl alcohol (PVA) was 98%.

Table 1 Composition of composite biomaterials CP/PLGA/tigecycline

Name	A_0	A_1	A_2	A_3
Tigecyclin (T) (wt%)	0.0	0.6	3.0	5.0
PLGA (wt%)	25.0	24.4	22.0	20.0
Calcium phosphate (CP) (wt%)	75.0	75	75.0	75.0

The calcium phosphate gel was added into completely dissolved polymer with different ratio of Tigecycline (Tygacil/Tigecyklin, Wyeth Europe Ltd., Berkshire, SL6 OPH) (according to Table 1). The suspension was mixed at a velocity of 18,000 rev/min, and then methanol was added. Afterwards, PVA (0.02% in water) was added into the suspension (PLGA/PVA = 10/1). Tigecyclin (T) content in the composite (given in Table 1) was obtained after calculating the encapsulation efficiency (%), according to the methodology known in literature [21], the encapsulation efficiency in our experiments was 70 ± 3%.

Ultrasonic deagglomeration was performed by *Sonics Vibra Sell*, High Intensity Ultrasonic Processor, (Ultrasonic Processors for High Volume Applications VCX 750 Newtown, Connecticut, USA, amplitude 80%). After that, the solution was centrifuged and decanted. The time and velocity of the centrifugal processing were 10 min and 4,000 rpm.

After the solvent evaporation, the particles were dried at room temperature for 24 h. The particles of calcium phosphate/PLGA composite biomaterial were sterilized by γ rays (25 kGy) before use. Table 1 shows the content of Tigecyclin after the preparation of composite and calculating the efficiency level.

2.2 Characterization of composite biomaterials

2.2.1 Infrared spectroscopy (FTIR)

The chemical composition of the composite material 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} .

2.2.2 X-ray (XRD)

X-ray structural (XRD) analyses were made using a Bruker D8 advance diffractometer equipped with focusing Ge

crystal primary monochromator that generates CuK_{α} radiation.

2.2.3 Dynamic Light Scattering (DLS)

Particle size distributions of CP/PLGA nanopowder were obtained using Brookhaven Instruments Light scattering system equipped with BI-200SM goniometer, BI-9000AT correlator, temperature controller and Coherent INOVA 70C argon-ion laser. Dynamic light scattering measurements were performed using 135 mW laser.

2.2.4 Atomic Force Microscopy (AFM)

Microstructural characterization was done by AFM (Thermo Microscopes, Autoprobe CP Research).

2.2.5 Ultraviolet (UV) spectroscopy

The release rate of Tigecycline in vitro in physiological solution (0.9% sodium chloride in water) was studied using UV spectroscopy. UV measurements were performed on PGBC UV–Visible Cintra 101 spectrophotometer in the frequency interval of 300–500 nm.

2.2.6 Cell cytotoxicity

The cellular cytotoxicity effect of CP/PLGA tigecycline on osteoblast cells was measured by the release of cytoplasmic lactate dehydrogenase (LDH) into the cell culture medium. Cell lines MC3T3-E1 were cultured in alpha-MEM supplemented with fetal bovine serum and penicillin and streptomycin. Cells were incubated at 37°C in a 5% CO_2 incubator and the medium was changed every other day. LDH activity of osteoblast cells were measured after 24 h, 48 h or 72 h. The LDH activity was measured by Cytotoxicity Detection Kit (Boehringer, Mannheim, Germany) according to manufacturer's protocol using 50 µl of the sample. The absorbance was measured with ELISA reader at 492 nm and reference filter at 620 nm.

2.2.7 In vivo study

In order to evaluate the antibiotic release kinetics in vivo, CP/PLGA loaded with 0.6% and 5%-antibiotic tigecycline samples were implanted into rats (Rattus norvegicus-albino). The Local Committee for Animal Studies of the University of Oslo-Norway had previously approved animal experiments. In vivo pilot study was used four animals in each group and results were presented as mean values. Complete in vivo results will be published in following study. Animals were anaesthetized with gas isofluran and implants were placed into muscle pouches in gluteus maximus of each rat. After 3 days animals were sacrificed and tissue surrounding implants, blood and kidneys from each animal were collected. The concentration of the antibiotic in samples was measured using high-performance liquid chromatography/tandem mass spectrometry (Series 200- Perkin Elmer and MS–MS API 2000 Applied Biosystems).

3 Results and discussion

The identification of all samples was carried out using infrared spectroscopy (Fig. 1a). Bands originating from CP, PLGA and Tigecycline (T) can be seen in the spectrum. CP is identified within the spectrum by a doublet 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 PO_4^{3-} group vibrations,

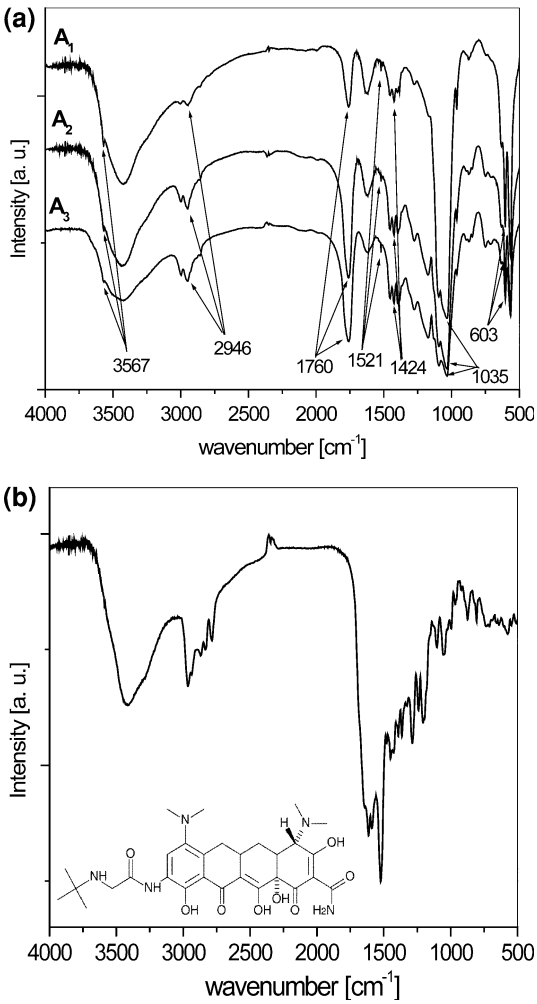

Fig. 1 FT-IR of **a** Nanocomposite of CP/PLGA with different ratio of tigecycline, **b** tygecycline

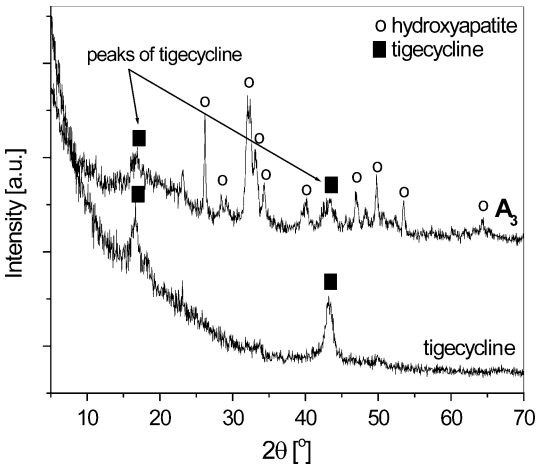

Fig. 2 XRD of tigecycline and CP/PLGA nanocomposite

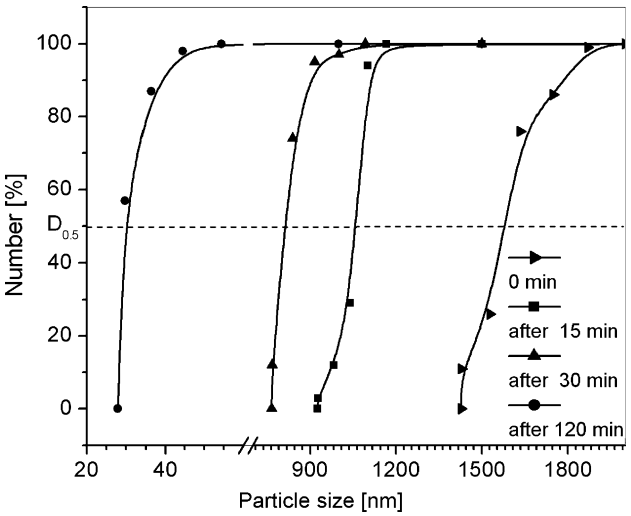

Fig. 3 Particle size distributions of the CP/PLGA powder ultrasonically deagglomerated during 0, 15, 30, and 120 min

and at 632 cm^{-1}, assigned to the hydroxyl group vibrations appearing also at 3,567 cm^{-1} [22]. The spectra indicate the presence of bands characteristic for PLGA. The band at 2,946 cm^{-1} corresponds to C–H ν_1 group. The intense, sharp band at 1,760 cm^{-1} is attributed to C=O vibration. The band at 1,424 cm^{-1} belongs to CH_3 group vibrations [23]. The band at 1,521 cm^{-1} corresponds to the most intense peaks from tigecycline $C_{29}H_{39}N_5O_8$ (Fig. 1b). The FT-IR spectroscopy data indicate that the obtained composite biomaterials A_1, A_2 and A_3 are made of CP, PLGA and tigecyclin antibiotic.

These results were confirmed by XRD testing. Diffraction spectra of CP/PLGA/T (A_3 specimens with maximum ratio of T) and pure T are shown in Fig. 2. The spectra have a part that is characteristic of CP [24]. The most intense peaks at 31.8° (2 1 1), 32.2° (1 1 2), 32.9° (3 0 0) and 49.5° (2 1 3) originate from calcium hydroxyapatite (HAp). XRD patterns show no peaks for PLGA, because it is an amorphous polymer, which is in accordance with the XRD studies of PLGA by other authors [23, 25]. The X-ray traces indicate poorly crystalline HAp. This may be the same as calcium-deficient apatite with non-stoichiometric Ca/P ratios lower than 1.67 observed by others [26]. This result was expected bearing in mind that the HAp gel was synthesized by precipitation at 100°C and pH = 12; HAp synthesis at this temperature leads to poorly crystalline HAp [26, 27]. The presence of tigecycline's characteristic peaks on composite's diffraction spectrum confirms the data obtained by FT-IR spectrometry indicating the presence of the antibiotic in the composite.

$D_{0.5}$ is known as the median diameter or the medium value of the particle size distribution; it is the value of the particle diameter at 50% in the cumulative distribution. A DLS analysis of the starting CP/PLGA powder (A_0) without antibiotic confirmed the highest value of $D_{0.5}$ equal to 1,580 nm (Fig. 3). Without additional processing and deagglomeration, an AFM analysis has shown that the obtained powder consisted of basic nanoparticles (NPs) in an agglomerated form [9]. The agglomerate consisted of basic nanoparticles joined together into bigger agglomerates by adhesive forces. After a 15-min ultrasonication, the $D_{0.5}$ of the agglomerates was 1,057 nm, whereas after a 30-min ultrasound treatment their $D_{0.5}$ reached 815 nm. A 120-min exposure time yielded non-agglomerated particles with $D_{0.5}$ equal to 30 nm, which acted as basic particles of the nanoparticulate composite biomaterial (Fig. 3).

The mechanism of particle deagglomeration in ultrasound field was studied in detail. Deagglomeration is a consequence of acoustic cavitation, inducing rapid liquid micro-jets. Microjets make waves which collide with a solid phase and physically separate it, i.e. perform deagglomeration of powder in liquids [28–30]. With the increase of ultrasonication exposure time from 15 to 30 and 120 min, under the same field frequency, the ultrasonic field power within the liquid CP/PLGA system also increases. The process leading to deagglomeration is thus expected to affect primary particles, too. Most probably, further increase in ultrasonication exposure time to over 120 min would bring about the delamination of PLGA on the surface of CP particles. Therefore, the adopted ultrasonication exposure time for further production of composite biomaterials with various contents of antibiotic (A_1, A_2 and A_3) is 120 min.

The morphology of the synthesised powder with different tigecycline content was analyzed by AFM (Fig. 4). In the study presented in this paper we used CP nanoparticles synthesized using the methodology that could ensure average particle diameter of approximately 30 nm [31], whereas that of pure CP/PLGA composite reaches around 40 nm [32]. Linear AFM analysis of the composite without

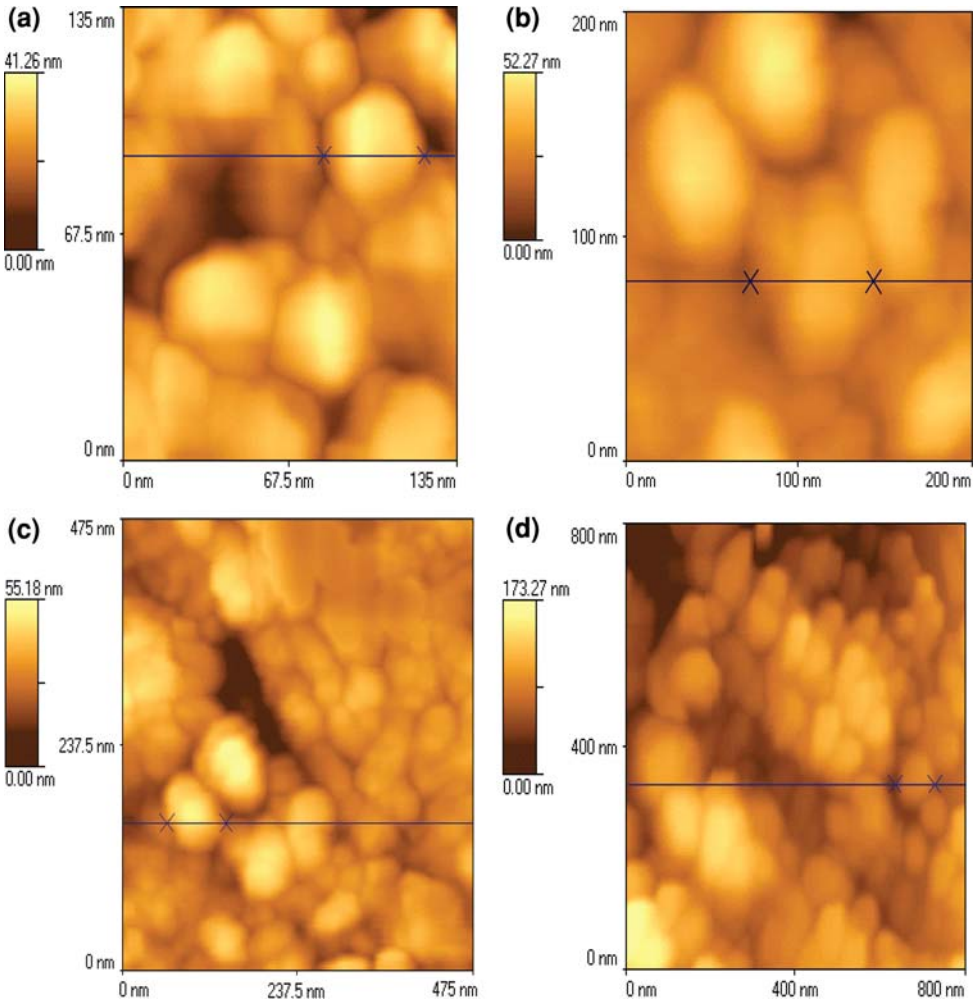

Fig. 4 AFM of CP/PLGA nanocomposite; **a** without tigecycline (A_0), **b** with 0.6% tigecycline (A_1), **c** with 3% tigecycline (A_2), **d** with 5% tigecycline (A_3)

tigecycline is shown on Fig. 4a and indicates an average particle diameter of around 40 nm. AFM morphology of the composite containing 0.6% of tigecycline is shown on Fig. 4b. It has been established by linear AFM analysis that the average particle diameter of this composite is around 65 nm. CP/PLGA/T composite with 3% of tigecycline (T) is shown on Fig. 4c. Its linear AFM analysis indicates an average diameter of around 80 nm. The obtained morphologies of the composites with tigecycline contents reaching 5% are shown on Fig. 4d; the average particle size is around 95 nm. Undoubtedly, the increase in tigecycline content within the composite leads to increased particle sizes. Similar phenomena were noticed in earlier studies in which PLGA nanospheres were used in controlled delivery of drugs and vitamins, where the diameter of PLGA spheres increased proportionally to the percentage of, for example, ascorbic acid. The procedure used for the synthesis of PLGA nanospheres with various contents of ascorbic acid was similar to ours [33].

During a 35-day period, whole tigecycline content is released within all systems. Figure 5 shows the dynamics of tigecycline release in physiological solution at 37°C under static conditions. In order to define release parameters, the curves shown in Fig. 5 were fitted to the most commonly used Peppas [19] and Weibull models [20]. The Peppas model (Eq. 1) was used for a mathematical description of drug delivery kinetics, where $M_t/M_\infty(\%)$ is cumulative release proportional to release constants k (h^{-1}) and n diffusional exponent characteristic of the release mechanism [19].

$$M_t/M_\infty = kt^n \quad (1)$$

The Peppas model or Peppas equation belongs to the group of empirical or semi-empirical mathematical models [34] which can be used to calculate diffusional exponent n that indicates transport mechanism (Table 2). Peppas model was used in modelling vancomycin release kinetics from microporous calcium phosphate ceramics [35].

The empirical Weibull (Eq. 2) function describes release profiles in relation to shape parameter, which defines transport mechanism (Table 3) [20, 35, 36].

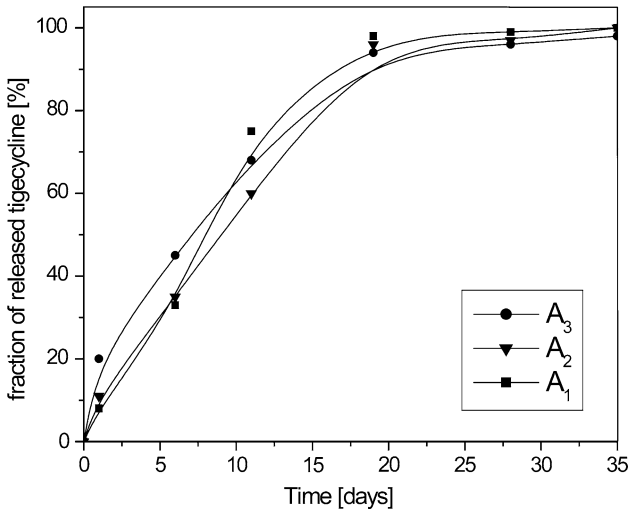

Fig. 5 Comparative cumulative curves of the release of tigecycline in percents over the degradation period

Table 2 Classification of the release mechanisms according to the release exponent n of the Peppas model [19]

Release exponent *n*			Release mechanism
Thin layer	Cylinder	Sphere	
$0.5 \leq n$	$0.45 \leq n$	$0.43 \leq n$	Fickian diffusion
$0.5 < n < 1.0$	$0.45 < n < 0.89$	$0.43 < n < 0.85$	Anomalous transport
$1.0 \geq n$	$0.89 \geq n$	$0.85 \geq n$	Case II transport

Table 3 Classification of release mechanisms according to the shape parameter *d* of the Weibull model

Shape parameter d	Release mechanism
$0.967 \leq d$	Fickian diffusion
$0.967 < d < 1.775$	Anomalous transport
$d \geq 1.775$	Case II transport

$$M = 100\left(1 - \exp(-x/\tau)^d\right) \quad (2)$$

In Eq. 2, x represents release time and τ time period during which 63.2% of the drug is released. M is cumulative release or $M_t/M_\infty(\%)$.

Release mechanism can be determined by correlating the obtained parameter of shape d from Weibull equation with other release parameters like diffusion exponent n from Peppas law [35]. Table 4 shows the results obtained by fitting the experimental results on Fig. 5 with Peppas and Weibull equations.

The obtained fitting results and classifications given in Tables 2 and 3 indicate that the release mechanism of the examined kinds of nanocomposite biomaterials (A_1, A_2 and A_3) under static conditions conform to anomalous transport mechanism. This kind of release mechanism is described in literature as non-Fickian transport [19]. It has been noticed that with the increase of tigecycline content, (from A_1 to A_3), release mechanism becomes close to Fickian diffusion. Non-Fickian transport is noticed when drug is released from thin polymer layers [37, 38]. Fitted experimental data related to drug release from a polymer film under in vitro conditions published by other authors show that the obtained release constant (k) and diffusional exponent (n) indicate a Non-Fickian transport [39]. Dominant phenomenon in cases of Non-Fickian transport is high-elastic stress in polymer related to relaxation time and it is non-linear [40].

Table 4 Fit parameters for the release of tigecycline from CP/PLGA nanocomposite biomaterials under static conditions according to the Peppas and Weibull models

Release from:	Peppas model		Weibull model
	n	*k* (h^{-1})	*d*
A_1	0.53 ± 0.12	3.05 ± 0.30	1.20 ± 0.09
A_2	0.50 ± 0.11	4.72 ± 0.38	1.13 ± 0.10
A_3	0.44 ± 0.06	6.23 ± 0.69	1.11 ± 0.09

Figure 6 also shows antibiotic release dynamics during 35 days for each system. After the first day (Fig. 6a–c) the highest release rate was achieved in system A_3 (20%), than in A_2 (11%) and the least in A_1 (8%). Most probably, 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. During first 20 days, accelerated release can be noticed in all systems. During 20 days 98% of the total amount of tigecycline is released in system A_1, 96% in A_2 and 94% in A_3.

LDH activity (citotoxicity) shows that CP/PLGA 5 wt% tigecycline (A_3) was higher at 24 h and 48 h time points ($P < 0.05$) compared to corresponding controls at each time point (Fig. 7). However, the LDH activity decreased over time for CP/PLGA 5 wt% tigecycline implants. Moreover, the cytotoxicty effect was under 40%, which is not considered high for this cell line. CP/PLGA 0.6 wt% tigecycline did not reveal any cytotoxic effect compared to corresponding controls. This is in agreement with other reports showing a positive effect of lower concentration of tetracycline on osteoblastic cells proliferation and increased mineralisation in the in vitro and in vivo model of normal bone metabolism [41, 42]. The concentrations MIC50 (250 ng/ml) and MIC90 (500 ng/ml) of tigecycline are know as minimal inhibitory concentrations for the most multi-resistant coagulase-negative staphylococci [43].

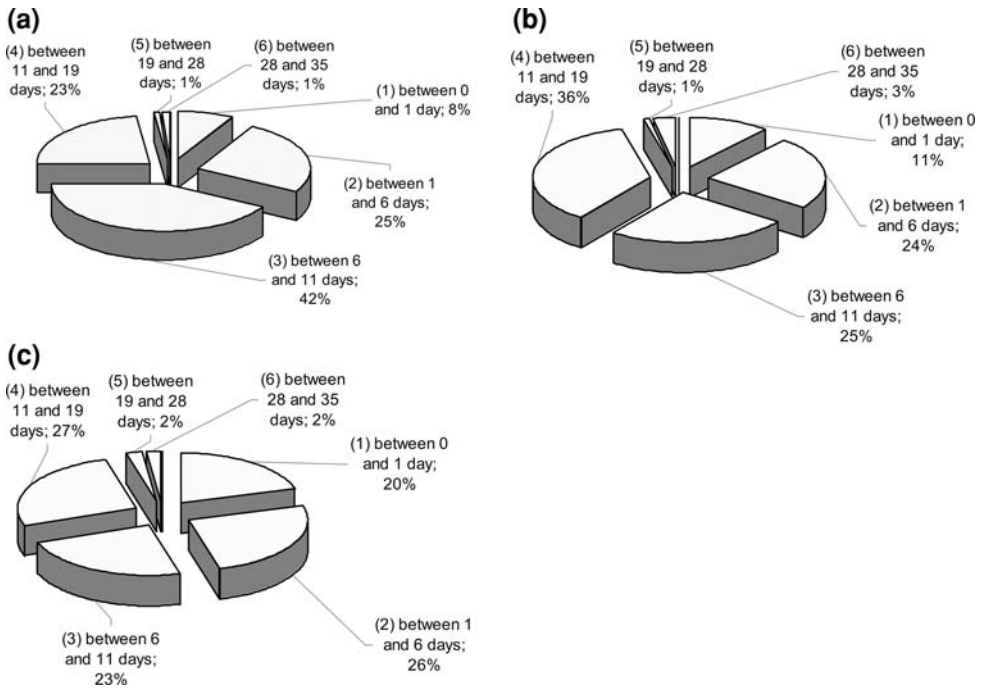

Fig. 6 Release of the tigecycline in percents over the degradation period: relative review for CP/PLGA/T **a** with 0.6% tigecycline (A_1), **b** with 3.0% tigecycline (A_2), **c** with 5% tigecycline (A_3)

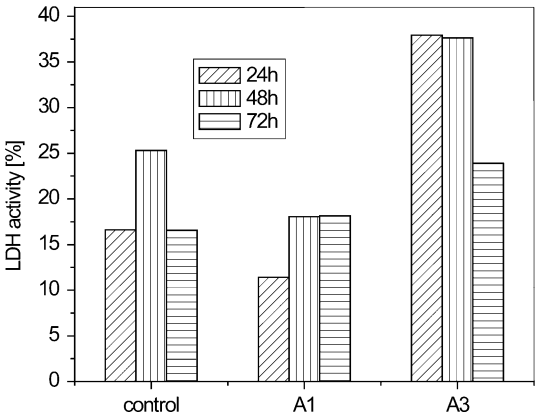

Fig. 7 LDH activity after 1, 2 and 3 days in medium with cell line MC 3T3-E1

Drug release from the system under real-life conditions can be studied only in living systems, where the release is influenced by numerous complex factors. The results of our in vivo study show that the implants with the lowest concentration of tigecycline (A_1) had 1,700 ng/g of tigecycline in the muscle surrounding the implant (A_1), which is more than threefold higher than MIC90 (500 ng/g) minimal inhibitory concentrations (Table 5).

Tigecycline was not found/measured in other muscles or kidneys after 3 days. The highest concentration of antibiotic (A_3) was 3,700 ng/g of tigecyline in the muscle surrounding the implant (A_3), which is more than sevenfold higher than MIC90 (500 ng/g) minimal inhibitory concentrations. The concentration of tigecycline in kidneys was 131 ng/g, whereas in the muscles of the other leg it reached 41 ng/g. According to the results obtained in the present study, the first system (A_1) shows the characteristics of a local controlled drug delivery system for the treatment of the most multi-resistant coagulase-negative staphylococci. However, system A_3 shows both high local and high overall/systemic concentration of tigecycline.

Table 5 Concentration of tigecycline after 3 days in different tissue of rats

Tissue		
CP/PLGA/T	A_1	A_3
Local surround tissue, close to biomaterial (ng/g)	1700	3700
Surround tissue, so far from biomaterial (ng/g)	0	395
Muscular of other leg (ng/g)	0	41
Kidney (ng/g)	0	131

4 Conclusion

Tigecycline-loaded nano composite biomaterial in which each calcium phosphate particle is coated with bioresorbable poly-DL-lactide-co-glicolide polymer, was synthesized. Nano biocomposite particles with 0.6, 3 and 5 wt% of tigecycline were synthesized by chemical bottom-up procedure. By linear AFM analysis that the average particle diameter of this composite varied from 65 to 95 nm. During a test under static conditions, the greatest amount of antibiotic was released during the first 20 days

of experiment, which was 98% for the system with 0.6 wt%, 96% for the system with 3 wt% and 94% for the system with 5 wt% of tigecycline. Kinetic release parameters indicating Non-Fickian transport during drug release were obtained by fitting the obtained experimental data according with the Peppas and Weibull models.

The measurement of the activity of lactate dehydrogenase, as a method to quantify cytotoxicity, after 1, 2 and 3 days showed values below 37%, indicating a very low level of cytotoxicity for this kind of composite biomaterial. As far as the use in living systems is concerned (rats, in our study), the composite with the lowest content of tigecycline proved to be suitable for local and controlled delivery of the antibiotic. Having in mind the registered antibiotics concentration in other tissues, delivery systems with a higher tigecycline content show both local and systemic effects.

Acknowledgements This study was supported by the Ministry of Science and Technological Development of the Republic of Serbia, under Grant No. 142006: Synthesis of functional materials with controlled structure on molecular and nano- levels. The authors would like to thank to Dr. D. Vasiljevic-Radovic for AFM analysis, Dr. M. Mitric for his kind help help in X ray measurements and Dr. M. Dramicanin for DLS analysis. The study is a result of joint research carried out by the Department of Biomaterials, Faculty of Dentistry, University of Oslo, under the guidance of Prof. Dr. Petter Lyngstadaas and Institute of Technical Sciences of the Serbian Academy of Sciences and Arts.

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