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Gentamicin loaded PLGA nanoparticles as local drug delivery system for the osteomyelitis treatment

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Since there are more and more cases of multiresistance among microorganisms, rational use of antibiotics (especially their systemic vs. local application) is of great importance. Here we propose polymeric nanoparticles as locally applied gentamicin delivery system useful in osteomyelitis therapy. Gentamicin sulphate (GS) was encapsulated in the poly(lactide-*co*-glycolide) (PLGA 85:15) nanoparticles by double emulsification (water/oil/water, $W_1/O/W_2$). The nanoparticles were characterized by dynamic light scattering, laser electrophoresis and atomic force microscopy. UV-vis spectroscopy (O-phthaldialdehyde assay, OPA) and Kirby-Bauer tests were used to evaluate drug release and antimicrobial activity, respectively. Physicochemical characterization showed that size, shape and drug solubilization of the nanoparticles mainly depended on GS content and concentration of surface stabilizer (polyvinyl alcohol, PVA). Laser electrophoresis demonstrated negative value of zeta potential of the nanoparticles attributed to PLGA carboxyl end group presence. Drug release studies showed initial burst release followed by prolonged 35-day sustained gentamicin delivery. Agar-diffusion tests performed with pathogens causing osteomyelitis (*Staphylococcus aureus* and *Staphylococcus epidermidis*, both reference strains and clinical isolates) showed antibacterial activity of GS loaded nanoparticles (GS-NPs). It can be concluded that GS-NPs are a promising form of biomaterials useful in osteomyelitis therapy.

Key words: *poly(lactide-co-glycolide), nanoparticles, gentamicin sulphate, ostemyelitis, Staphylococcus aureus, Staphylococcus epidermidis*

List of abbreviations

AFM – atomic force microscopy
DLS – dynamic light scattering
GS – gentamicin sulphate
GS-NPs – gentamicin sulphate loaded nanoparticles
MIC – minimal inhibitory concentration
OPA – O-phthaldialdehyde
PBS – phosphate buffered saline
PLGA – poly(lactide-*co*-glycolide)
PVA – polyvinyl alcohol
UHQ-water – ultra high quality water
$W_1/O/W_2$ – water-oil-water emulsification
$Zr(acac)_4$ – zirconium acetylacetonate
%EE – encapsulation efficiency
%LE – loading efficiency

1. Introduction

Surgical actions are accompanied by the risk of microbial infections, independently of the tissue affected. In the case of bone, such infections often result in serious skeletal tissue damage and furthermore in the spreading of the microbes to the adjacent tissues and organs [16]. As a result of microbes abundance a chronic inflammation arises that utmost concerns tibia, femur, clavicle [24]. The developing onset of symptoms is called osteomyelitis [24]. The consequence of osteomyelitis is chronic inflammation, abscesses formation, sinus

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appearance, severe pain, and finally tissue necrosis [24].

One of the possible strategies to treat osteomyelitis is aminoglycoside antibiotic administration. Gentamicin sulphate (GS) is here utmost used agent that deals with severe infections caused by Gram-positive (e.g., *Staphylococcus* spp.) and also by Gram-negative bacteria. GS, acts by irreversible hindering bacteria 30S ribosomal subunit and thus causes misreading of bacterial t-RNA molecule. Contact with antibiotic makes the microorganisms incapable to synthesize proteins essential to their growth and thus prevents showing up or spreading of the infection [25]. Recently, there are two main procedures applied in order to struggle with osteomyelitis. The first approach implies intravenous injections by an indwelling catheter, applied three times a day [15], whilst the second is based on locally placed bone cement or collagen sponges saturated with GS [22]. Since success of the antyosteomyelitic therapy firmly depends on the proper doses of antibiotic availability, administered closely at the infected site that should last at least up to 6 weeks, a very promising solution seems to be encapsulation of antimicrobial drug into slow degrading biomaterial which is thought to, after being placed directly inside the bone, delay gentamicin release to appropriate rate, dose and time [4]. Poly(lactide-*co*-glycolide) (PLGA) was chosen to encapsulate the drug, because this FDA-approved resorbable copolymer has a tunable degradation rate (i.e., by molar ratio of lactide to glycolide, chain structure, molecular weight) [15]. PLGA has already been used for the production of sutures, osteosynthesis devices, scaffolds for bone tissue regeneration and drug delivery systems [15], [19].

Thus, the main objective of our study was to prepare and optimize in terms of size and solubilization a new drug delivery system containing gentamicin. The system proposed was based on PLGA 85:15 and was produced by the double emulsification technique. After preparation, the detailed physicochemical characterization of the nanoparticles was performed, and the most highly loaded drug delivery systems we evaluated in context of GS release kinetics and their antimicrobial properties.

2. Materials and methods

2.1. Materials

Gentamicin sulphate (Gentamicini sulfas, $C_{21}H_{43}N_5O_7 \cdot H_2SO_4$) was obtained from Galpharm, Poland while polyvinyl alcohol (PVA, Mowiol 4-88, Mn = 31,000 Da), phosphate buffered saline (PBS) were obtained from Sigma-Aldrich, Poland; dichloromethane, methanol, mercaptoethanol, isopropanol, orthophthaldialdehyde (OPA) were all of analytical grade and were purchased from POCh, Poland. Ultra high quality water (UHQ-water) was produced in UHQ-PS purification system (Elga, UK). Poly(lactide-*co*-glycolide) (PLGA, 85:15, Mn = 80 kDa, d = 1.9) was synthesized in the Centre of Polymer and Carbon Materials of the Polish Academy of Sciences in Zabrze, Poland by a ring-opening polymerization in bulk at 100 °C with zirconium acetylacetonate ($Zr(acac)_4$) initiator [20], [21].

2.2. Preparation of the nanoparticles

Nanoparticles loaded with gentamicin sulphate (GS-NPs) were prepared by means of a water-oil-water ($W_1/O/W_2$) emulsification solvent evaporation method. Firstly, an oily phase (O) was prepared by dissolving 100 mg of PLGA in 6 ml of dichloromethane (1.67% w/v). Secondly, internal W_1/O emulsion was prepared by dispersing GS solution (2, 10 or 20 mg in 100 μl of UHQ-water and 45 μl of 4% w/v PVA) within an oily phase during ultrasonication (3 min, Sonics VibraCell™, USA, 40% of the cycle) on ice. In order to form double emulsion $W_1/O/W_2$, obtained earlier W_1/O emulsion was added drop by drop to 20 ml of 1%–4% (w/v) PVA solution (external water phase, W_2) in conditions as mentioned above. Formed double emulsion $W_1/O/W_2$ was left overnight on mechanical agitator (1000 rpm) in order to allow dichloromethane evaporation. Formatted colloidal dispersion of loaded carriers was further centrifuged (14,000 rpm, 4 °C, 20 min, MPW 351R, Medical Instruments, Poland) and three times flushed with UHQ-water. Afterwards, a portion of nanoparticles was freeze-dried and then stored at 4 °C. Before further assessments such as size, surface charge, morphology the particles were redispersed in UHQ-water using ultrasonic bath (POLSONIC® SONIC-3, 10 min).

2.3. Physicochemical analysis of nanoparticles – size and zeta potential

The size as well as polydispersity index of obtained nanoparticles were determined with the use of dy-

namic light scattering method (DLS) on Zetasizer Nano ZS (Malvern Instruments) equipped with He-Ne laser 632.8 nm. To perform the measurements the scattering angle 173° was set and correlator ALV 500 was engaged. Size values were determined according to Stokes-Einstein equation. Zeta potential was measured by laser Doppler electrophoresis method on the same apparatus and calculated using the Smoluchowski equation out of electrophoretic mobility data performed in triplicate for every sample.

2.4. Morphology of the nanoparticles

Morphological evaluation of nanoparticles was performed on atomic force microscope (AFM). Nanoparticles suspension was placed on microscopic glass and water was allowed to evaporate for 12 h at room temperature. Topographic images were recorded in air using Si_3N_4 tips with a nominal radius of curvature of 30 nm (NanoProbeTM tips) in contact mode on AFM (Explorer, Thermomicroscopes, Veeco). A feedback mechanism was employed to adjust the tip-to-sample distance to maintain a constant force of 0.1 nN between the tip and the sample with proportional, integral and derivative parameters of 1, 0.3 and 0, respectively. All the images were flattened and treated using the software SPMLab6.02.

2.5. Solubilization of the antibiotic

In order to assess an amount of GS that actually remained enclosed within the nanoparticle structure, supernatant out of particles centrifugation was collected. A GS content was examined spectrophotometrically with the use of an OPA test [1] in the spectrophotometer (UV-VIS Cecil CE 2502, Corston UK) at 332 nm. A standard calibration curve was plotted in appropriate PVA concentration within range 5–400 µg/ml of the drug. Parameters referring encapsulation efficiency (%EE) and loading efficiency (%LE) were calculated according to the formulas

$$\%\ \textit{Encapsulation Efficiency} = \frac{\textit{mass of GS in nanoparticles}}{\textit{initial mass of GS in the system}} \cdot 100\%, \quad (1)$$

$$\%\ \textit{Loading Efficiency} = \frac{\textit{mass of GS in nanoparticles}}{\textit{mass of nanoparticles}} \cdot 100\%. \quad (2)$$

All experiments were performed in triplicate and the results were expressed as mean ± standard error of the mean (S.E.M.).

2.6. Release study

The release profile of GS from GS-NPs suspended in PBS buffer was analyzed. End-sealed dialysis bags (ZelluTransRoth, MWCO 12 kDa) were filled with 1 ml of the suspension (10 mg of the GS-NPs prepared in the following conditions: 4% w/v PVA and 20 mg of GS per 100 mg PLGA which in total contained 1 mg of drug) and immediately immersed in the vials with 20 ml of PBS at pH 7.2. Then the vials were placed on mechanical stirrer (50 rpm) at 37 °C. At predetermined time intervals 2 ml of PBS that contained released antibiotic were collected and analyzed in terms of the drug content (the aliquots that were withdrawn from the system were replaced with 2 ml of fresh PBS). To determine antibiotic amount an OPA assay according to the method described by Anhalt et al. [1] was used. The results were expressed as amount of released antibiotic with respect to time passage.

2.7. Antimicrobial activity

Kirby-Bauer method (Agar diffusion test) was applied in order to determine antimicrobial activity of GS-NPs. 30 mg of the GS-NPs (produced in the following conditions: 4% w/v PVA, 20 mg of GS/100 mg PLGA) were mixed with 0.5 ml of PBS buffer pH 7.4 and shaken overnight on vortex at room temperature. After centrifugation the aliquots were gathered and zone inhibition test against two main bone-related Gram-positive pathogens *Staphylococcus aureus* and *Staphylococcus epidermidis* was performed. The reference strains used in the study were *S. aureus* DSM 24167 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and *S. epidermidis* ATCC 700296 (American Type Culture Collection). The clinical isolates of *S. aureus* (SA1-KCR) and *S. epidermidis* (SE1-KCR) were isolated from patients with joint infection hospitalized at the Kraków Centre of Rehabilitation and Orthopedics in 2012. The used strains were incubated in 5 mL of Bacto™ Tryptic Soy Broth (Becton Dickinson) for 16 h at 37 °C and prepared in concentration 0.5 in McFarland scale (1.5×10^8 CFU/ml) in physiological saline solution. Then they were seeded on Mueller-Hinton agar (Difco) plates in which 3 mm

wells were cut and in these wells 50 μl of aliquots were placed. As a reference 10 μg gentamicin standards (Oxoid, UK) were used. The plates were then incubated at 37 °C for 18 h and the zone of microbes growth inhibition was measured using Calibrating Viewer [mm]. The experiment was performed in triplicate.

3. Results

3.1. Physicochemical properties of nanoparticles

The results of size, zeta potential, polydispersity index and solubilization parameters of GS-NPs are shown in Table 1. PLGA GS-NPs were formulated with an average size of 200–400 nm. Nanoparticles were also of a low polydispersity, ca.0.3, which corresponded to a uniform size distribution. The zeta potential was in the range –7.2 mV to –1.1 mV. The solubilization efficiency was in the range 2.7–52.2%, and was the highest for the highest GS amount (drug to polymer ratio 20:100) as well as the surfactant concentration (4%). Under these conditions loading efficiency was also highest (up to 10.3%).

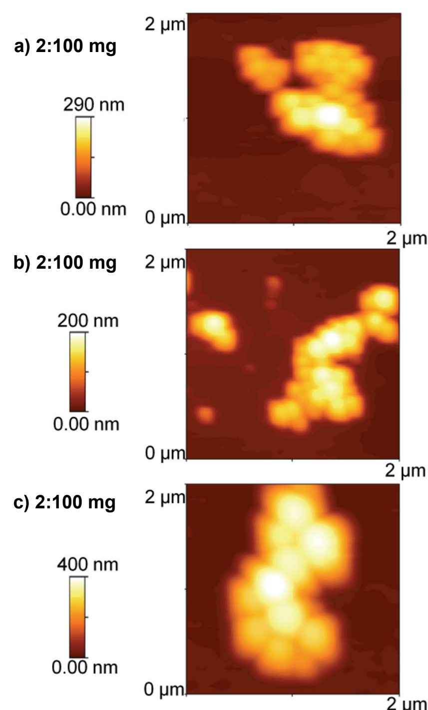

Fig. 1. AFM images of GS-NPs: drug to polymer ratio 2:100 mg and PVA 1% (a), drug to polymer ratio 2:100 mg and PVA 4% (b); drug to polymer ratio 20:100 and PVA 4% (c); contact mode, 2 μm × 2 μm

Table 1. Size, zeta potential, polydispersity indices and solubilization parameters of GS-NPs for different preparation conditions

Composition		D_H	PdI	ζ	Solubilization parameters	
GS concentration [mg/100 mg of PLGA]	PVA concentration [%]	[nm]	[–]	[mV]	% EE	% LE
2 mg	1%	307 ± 8	0.38 ± 0.04	–7.2 ± 1.0	2.7 ± 1.8	0.06 ± 0.05
2 mg	2%	219 ± 11	0.27 ± 0.03	–1.1 ± 0.1	11.2 ± 1.5	0.34 ± 0.02
2 mg	3%	214 ± 4	0.24 ± 0.01	–5.1 ± 0.5	21.3 ± 1.8	0.31 ± 0.01
2 mg	4%	222 ± 37	0.30 ± 0.02	–3.6 ± 0.2	15.6 ± 0.9	0.37 ± 0.01
10 mg	4%	234 ± 11	0.21 ± 0.01	–5.2 ± 0.5	45.7 ± 1.0	7.25 ± 0.06
20 mg	4%	391 ± 23	0.346 ± 0.002	–3.5 ± 0.3	52.4 ± 0.5	10.28 ± 0.04

D_H – hydrodynamic diameter, PdI – polydisperisty index, ζ – Zeta potential, %EE – encapsulation efficiency, %LE – loading efficiency.

3.2. Morphology of the nanoparticles

AFM images demonstrated that the nanoparticles were spherical and uniform in shape (Fig. 1). The size of the particles decreased with increase in surfactant concentration (Fig. 1a vs. b). The nanoparticles loaded with the highest GS amount (drug to polymer ratio 20:100, PVA 4%) were the biggest (Fig. 1c).

3.3. Drug release study of the nanoparticles

GS release profile from nanoparticles suspended in PBS buffer and images of such nanoparticles in the test-tubes are shown in Figs. 2a and 2b, respectively.

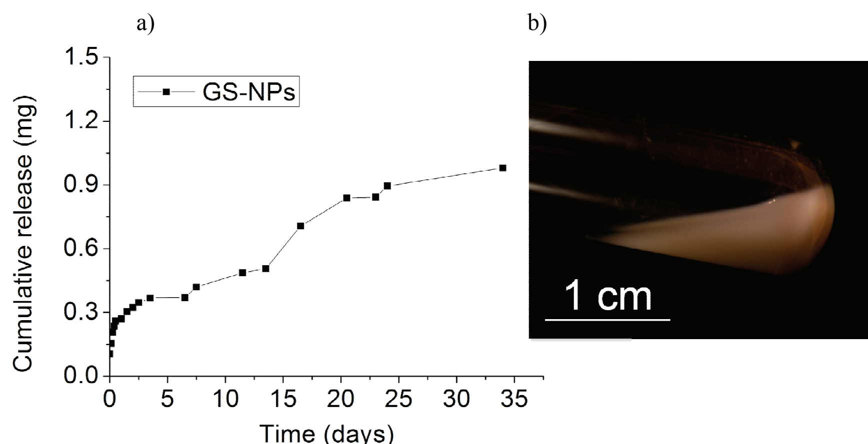

Fig. 2. Drug release study from gentamicin loaded nanoparticles (GS-NPs) (a) and morphology of the sample in the test tube (b)

The sample was liquid, turbid and milk-white. The burst release of the drug was observed. It was found that 25% of the loaded drug was released in the first 12 h. Next, a sustained drug release phase was observed that continued up to day 35, when the entire amount of encapsulated drug (1 mg) had been released.

3.4. Antimicrobial activity

In Fig. 3, the plates with the tested strains of staphylococci grown for 18 h with GS-NPs aliquots are shown. Around wells that contained GS-NPs aliquots the zone of growth inhibition was observed, with diameters in the range 32–39 mm depending on the pathogen type; precise values of the diameters of the growth inhibition zones are shown in Fig. 4. For 10 µg standards of gentamicin the zones of growth inhibition were 27 mm (for *S. aureus* DSM 24167 and *S. epidermidis* SE1-KCR) and 28 mm (for *S. epidermidis* ATCC 700296 and *S. aureus* SA1-KCR) (data not presented).

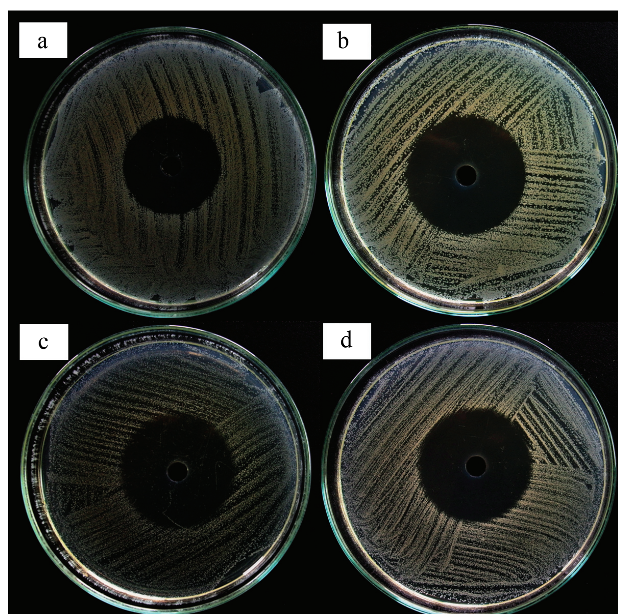

Fig. 3. Antimicrobial activity of GS-NPs aliquots. Inhibition zone of: the reference strains of *S. aureus* DSM 24167 (a), the clinical isolate of *S. aureus* SA1-KCR (b), the reference strains of *S. epidermidis* ATCC 700296 (c) and the clinical isolate of *S. epidermidis* SE1-KCR (d)

4. Discussion

4.1. Characteristics of gentamicin-loaded nanoparticles

In this study, GS-NPs with diameter of 200–400 nm were produced. It was achieved by utilization of PLGA solution of low concentration (1.67%) in W_1/O phase. Such a concentration resulted in low viscosity of the W_1/O, which enabled it to be dispersed better during the second emulsification stage (W_2) as shown previously [18]. Surfactant amount, acting in the experiment as an interphase stabilizer, also had a significant impact on the size of developing particles. When higher concentrations of PVA (2%, 3% or 4% w/v) were used, the size of the particles decreased. It also resulted in a decrease in polydispersity index as previously reported by others [10]. The AFM analysis confirmed the sphericity and narrow size distribution of the nanoparticles. Determination of size by DLS measurements and AFM visualization yielded similar results, although the light scattering technique provides the values derived from hydrodynamic meas-

urements, while AFM provides data for the dried samples. It can be anticipated that the obtained nanoparticles with such a size will remain within the extracellular space of bone tissue. It was reported so far that too small carriers (<100 nm) tend to be endocytosed, while too big carriers (>500 nm) undergo phagocytic uptake [23].

The nanoparticles demonstrated negative zeta potential values (–7.2 mV to –1.1 mV), which can be attributed to the presence of the ionized carboxyl group of PLGA on the particles' surface. Interestingly, pure PLGA showed a much lower zeta potential (–30 mV to –50 mV) [3], which suggests that PVA molecules adsorb on the surface of the nanoparticles. A similar size and zeta potential values were also reported by Grabowski et al. who studied PLGA nanoparticles obtained with the use of PVA and attributed such values of zeta potential to the remnants of surfactant [12].

Regarding solubilization, it was observed that both encapsulation and loading efficiencies significantly increased with the surfactant concentration. This can be explained by the fact that smaller droplets developed when the PVA concentration was higher and smaller particles exposed a higher relative surface area. As a consequence such particles solidified faster because the solvent evaporated more rapidly. Hence the escape of the phase containing the drug (W_1) to the external water phase W_2 was prevented. Similar enhancement of solubilization with increased PVA concentration has already been described in other studies [6].

Additionally, both solubilization parameters were increased by higher drug concentration in the W_1 phase. It can be concluded that changes in surfactant concentration and the drug amount are the factors that finely tune the solubilization parameters as well as particle size during formation of GS-NPs.

4.2. Drug release kinetics

Systemic drug infusions of GS (applied as general way of treating osteomyelitis) are known to provoke nephro- and ototoxicity [11]. On the other hand local treatment of osteomyelitis with the use of GS loaded cements or collagen sponges requires traumatic surgical procedure [7]. Thus recently great attention is paid on local tissue-directed ways of antibiotics administration via injections. Effective intra bone osteomyelitis treatment requires maintenance a therapeutic antibiotic concentration for at least 3–4 weeks [20]. In our drug release studies from GS-NPs we observed immediate drug release phase accounting for ca. 25% of the whole GS dose embedded in the nanoparticles followed by prolonged drug release phase up to 35 days. Such shape of release curve is typical of diffusion – erosion controlled mechanism [17]. According to Jain et al. [13], immediate release phase can be ascribed to release of surface-associated amount of antibiotic driven by diffusion. Next stage of drug release originated from hydrolytic bulk degradation of the polymer which caused diffusion of oligomers out of the nanoparticle that created microcavities in the particles and facilitated drug migration [13]. Initial burst of drug dose followed by its sustained release is in compliance with recommendations of gentamicin use [11].

4.3. Antimicrobial activity against staphylococci

The antimicrobial effect of the GS-NPs was evaluated by observation of growth inhibition zones of *S. aureus* and *S. epidermidis* strains in contact with aliquots containing GS-NPs. Such pathogens were chosen because they are mainly responsible for intra bone infection development leading to osteomyelitis: 34% and 32% of reported cases (totally 2/3 of the whole cases) are caused by *S. aureus* or by *S. epidermidis*, respectively [5]. In our studies (see Fig. 3) we observed that by application of GS-NPs suspension we succeeded with stopping growth of staphylococci species. The diameters of the inhibitions zones after 18 h of incubation were between 32–39 mm and 39–37 mm (see Fig. 4) for *S. aureus* and *S. epidermidis*, respectively. In general for the *S. epidermidis* type higher diameters range were obtained than for *S. aureus*. The observations

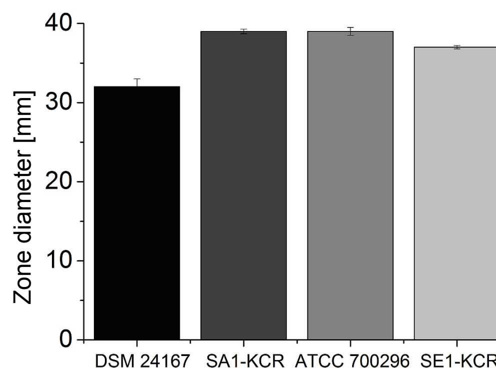

Fig. 4. Diameters of growth inhibition zones of staphylococci strains in contact with GS-NPs; the reference strain of *S. aureus* DSM 24167, the clinical isolate of *S. aureus* SA1-KCR, the reference strain of *S. epidermidis* ATCC 700296 and the clinical isolate of *S. epidermidis* SE1-KCR

correspond to minimal inhibitory concentration values (MIC) of GS for both species: for *S. epidermidis* MIC (0.064 μg/ml) is lower than for *S. aureus* (0.500 μg/ml) [14].

Resistance to antibiotics is nowadays one of the mayor concerns in the antimicrobial therapy. Often the reference strains of microorganisms are more susceptible to antibiotics whereas for clinical isolates the response is reduced [5]. Clinical staphylococci species can be methicillin/oxacillin, aminoglycosides, macrolides, lincosamides, tetracyclines, trimethoprim or sulfonamides resistant [2]. In conducted studies both types of microorganisms, e.g. reference strains and clinical isolates were analyzed. For all tested conditions significant inhibition zones were noted which overall proved broad antibacterial activity of GS-NPs. According to the European Committee on Antimicrobial Susceptibility Testing from 01.01.2014 (EUCAST) zone diameter breakpoint for gentamicin [10 μg] for *S. aureus* is 18 mm, and for coagulase-negative staphylococci eg. *S. epidermidis* is 22 mm [9]. Based on these recommendations it can be concluded that the procedure of GS-NPs manufacturing did not affect antibiotic activity of the encapsulated drug and GS-NPs seem to be promising drug delivery system in the treatment of osteomyelitis.

5. Conclusion

Based on the results obtained we conclude that by encapsulation of GS in PLGA nanoparticles it was possible to fabricate prospective drug delivery systems for bone infection treatment. By changing manufacturing parameters (GS content in relation to PLGA and PVA concentration) it was possible to adjust size and solubilization parameters of GS-NPs. Short burst release followed by long sustained release of antibiotic was obtained up to 5 weeks. Drug released from GS-NPs demonstrated antimicrobial activity against reference strains and clinical isolates of *S. aureus* and *S. epidermidis*, which are responsible for majority of osteomyelitis cases.

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