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Efficiency of gentamicin loaded in bacterial polysaccharides microcapsules against intracellular Gram-positive and Gram-negative invasive pathogens

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Abstract

Gentamicin is an aminoglycoside antibiotic with a wide spectrum of anti-bacterial activity, but however, due to its high solubility in water, it poorly penetrates inside the cells. This major inconvenient constitutes an important challenge for the treatment of intracellular bacterial infections, which might be solved using appropriate delivery systems for the targeted release of the bioactive agents at the intracellular sites of infection. Thus, in the case of antibiotics, the use drug delivery systems may contribute to increase their therapeutic activity against intracellular pathogens. This paper presents an efficient polymeric delivery system for the intracellular release of gentamicin based on bacterial polysaccharides.

Keywords: drug delivery, bacterial polysaccharides, microspheres, gentamicin.

Introduction

Biodegradable polymers have been extensively studied for the fabrication of drug delivery systems over the past few decades [1]. The most studied polymers for such applications are poly(D,L-lactide), poly(lactic acid) (PLA), poly(D,L-glycolide) (PLG), poly(D,L-lactide-*co*-glycolide) (PLGA), and poly-cyanoacrylate (PCA) [2].

Delivery systems based on polysaccharides are designed for the release of the drug at the site of action for a long period of time, so that therapeutic doses can be reduced considerably [3]. On the other hand, different forms of delivery systems are able to reduce fluctuations of drug substance concentrations in plasma, in order to obtain an effective pharmacological response [4]. The usage of targeted delivery systems could allow the achievement of a controlled and prolonged release of some medications, for maximum therapeutic efficiency and minimal side effects.

Limited cellular penetration reduces the effectiveness of many antimicrobial treatments, such as tetracyclines, cephalosporins, penicillins and aminoglycosides [5].

Gentamicin exhibits bactericidal activity against a broad spectrum of microorganisms including *Pseudomonas* (*P.*) *aeruginosa*, *Staphylococcus* (*S.*) *aureus* and some members of *Enterobacteriaceae* family [6]. Similar to other aminoglycoside antibiotics, gentamicin exhibits concentration-dependent bactericidal activity and a prolonged post-antibiotic effect [7]. Because of its polar nature, the oral absorption rate and the tissue penetration of gentamicin is poor and it is excluded from most cells [7]. In this case, appropriate drug delivery systems may increase the therapeutic activity of antibiotics in intracellular locations [8]. Until now, it was proved that many of the drug delivery systems, based on biodegradable polymers (*e.g.*, PLGA, chitosan, polybutylcyanoacrylate) improved the intracellular uptake of different antibiotics, such as isoniazid [9], cefotaxime [5], streptomycin [10], or gentamicin [11–15]. Prior *et al.* demonstrated the effectiveness of chitosan in the intracellular release of tetracycline against *S. aureus* [16].

Efficiency of polysaccharidic polymers in delivering intracellular pharmacologically active compounds has been extensively studied also in relation to the anti-tumoral drugs [17, 18] and non-viral transfection [19].

These studies demonstrated the effectiveness of polysaccharide polymers such as developing new carrier molecules for the transport and controlled release and targeted antibiotics.

The purpose of this paper was to investigate the efficiency of some new delivery systems based on

bacterial polysaccharides extracted from *Klebsiella* and *Pseudomonas aeruginosa* strains loaded with gentamicin against intracellular pathogens.

Materials and Methods

Microbial strains

For exopolysaccharides fraction extractions, clinical isolates of *K. pneumoniae* and *P. aeruginosa* strains were used. The strains were isolated from patients with different diseases hospitalized between 2009–2011 at the "Prof. Dr. Constantin C. Iliescu" Emergency Institute for Cardiovascular Diseases, Bucharest, Romania.

The efficiency of polysaccharidic microcapsules loaded with gentamicin on intracellular bacterial pathogens was studied against eight *L. monocytogenes*, one *P. aeruginosa* and seven *E. coli* strains from the collection of the Department of Microbiology, Faculty of Biology, University of Bucharest, previously tested for their invasive properties.

Drug delivery design

The exopolysaccharidic fractions were extracted from *K. pneumoniae* (K742, K800) and *P. aeruginosa* (P568) strains using Ojea-Jiménez *et al.* [20] modified method and characterized by FTIR (Fourier transform infrared spectroscopy), SEM (scanning electron microscopy) and XRD (X-ray diffraction) according to our previously published paper [21]. For polysaccharidic microcapsules loading, gentamicin was adsorbed on the polysaccharidic support by co-solubilization, followed by grinding the obtained material after evaporation of the solvent at a final concentration of 10% (100 mg of polysaccharides mixture contains 90 mg polysaccharides and 10 mg of antibiotic). Polysaccharides and gentamicin were mixed with 2 mL of ultrapure water until its complete evaporation at 40^0C. Hybrid systems were refrigerated until use.

The invasion capacity assay was performed by Cravioto's adapted method [22, 23] for the quantification of the intracellular, invasive bacteria after the removal of the extracellular ones. Briefly, the HeLa cell monolayers (at 70–80% confluence) grown in 6-well plates (Nunc) were washed three times with phosphate-buffered saline (PBS) and 1 mL of fresh DMEM (Dulbecco's Modified Eagle's Medium, Sigma) without antibiotics was added to each well. The tested strains grown on solid medium were suspended in PBS to an optical density of 0.5 McFarland (~10^8 colony-forming units (CFU)/mL) and 1 mL was used to inoculate three wells/strain. The inoculated plates were incubated for two hours at 37^0C. After the incubation period, one sample for each strain was treated with 500 μL/well of 100 μg/mL gentamicin solution prepared in PBS, in order to kill the extracellular bacteria, and the second well with 500 μL/well of 100 μg/mL gentamicin encapsulated in the bacterial polysaccharidic microcapsules. The third set of wells was maintained in standard conditions (in the initial culture medium). The plates were further incubated for another hour in the same conditions. After incubation, plates were washed three times with PBS and permeabilized with 0.1% Triton X-100 (Sigma) for five minutes at 37^0C. Serial dilutions of suspended cells harvested from the plate wells were seeded on solid media (casein soy agar) (three technical replicates/dilution) in order to establish the invasion indexes (CFU/mL).

Fluorescence microscopy

Eukaryotic cells modifications induced by the bacterial polysaccharides loaded with gentamicin were highlighted by fluorescent staining with propidium iodide (PI). The obtained preparations were visualized by fluorescence microscopy (AxioLab, Carl Zeiss) using the immersion objective (×100) and wavelength channel of 546 nm (red).

Results

The purpose of this study was to evaluate the effectiveness of polymeric microcapsules loaded with the gentamicin antibiotic using a culture-based method for assessing the bacterial invasion in the presence of gentamicin alone and encapsulated drug. The FTIR spectroscopy analysis performed comparatively on the polysaccharides and respectively, on microcapsules loaded with gentamicin has proved that the antibiotic was loaded successfully, without affecting its structure, while scanning electron microscopy images revealed the homogeneity of the microcapsules that do not exceed 30 μm [20].

Gram-positive and Gram-negative bacterial strains of *L. monocytogenes*, *E. coli*, *P. aeruginosa* previously characterized for their invasion capacity were analyzed using quantitative and qualitative methods. The tested bacterial strains succeeded to invade the cellular substratum with different rates, but in the presence of gentamicin encapsulated in the polymeric microcapsules, the invasive viable cell counts decreased significantly, which demonstrates that by encapsulation, gentamicin, an antibiotic acting on extracellular bacteria, can be "delivered" inside of eukaryotic cells, becoming efficient on internalized bacteria (Figures 1–3).

All tested microcapsules encapsulated with gentamicin were efficient against the analyzed strains. Moreover, a total suppression of invasion capacity in the presence of gentamicin incorporated in K742, K800, P568 polysaccharidic microcapsules was found in 53.5%, 61.5% and respectively, 80% of the analyzed strains. The K742 and K800 microcapsules loaded with gentamicin totally inhibited the invasion capacity of four *Listeria* sp. and three *E. coli* strains (Figures 1 and 2), while P568 blocked the invasion of *Listeria* sp. strains (Figure 3).

The internalization of gentamicin-loaded microcapsules into the mammalian cells was also sustained by the cellular morphology changes produced by the bacterial polysaccharides on HeLa cells, revealed by fluorescence staining. The presence of polysaccharides induced fine cytoplasmic extensions, a consequence of cellular events, as actin cytoskeleton reorganization and the presence of vacuoles in the cytoplasm, witnessing the stimulation of endocytosis (Figures 4 and 5).

These data revealing the entrance of microcapsules into the cells, and corroborate with the reduced number of invasive viable cell counts, demonstrating the effectiveness of the encapsulated gentamicin against intracellular microorganisms.

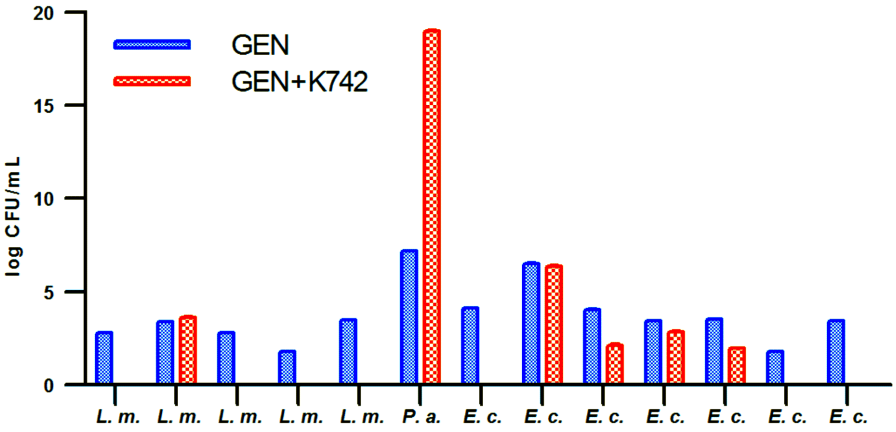

Figure 1 – *The graphic representation of the effectiveness of gentamicin incorporated in microcapsules of K. pneumoniae 742 polysaccharide versus gentamicin solution. L.m.: Listeria monocytogenes; P.a.: Pseudomonas aeruginosa; E.c.: Escherichia coli.*

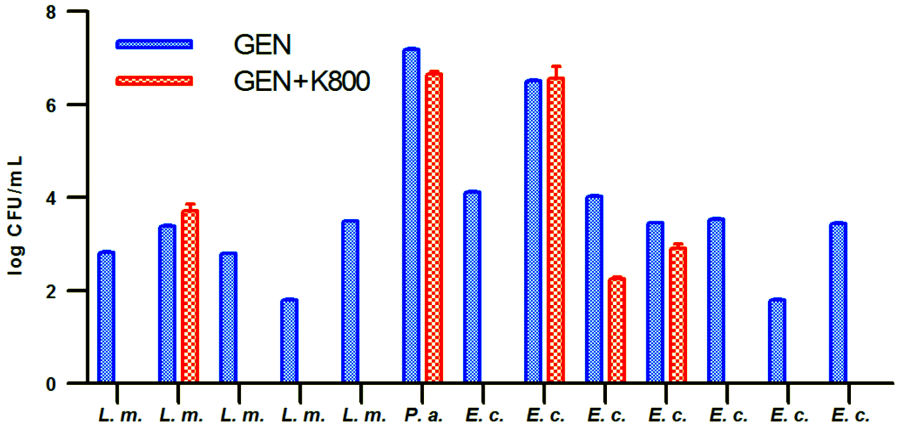

Figure 2 – *The graphic representation of the effectiveness of gentamicin incorporated in microcapsules of K. pneumoniae 800 polysaccharide versus gentamicin solution. L.m.: Listeria monocytogenes; P.a.: Pseudomonas aeruginosa; E.c.: Escherichia coli.*

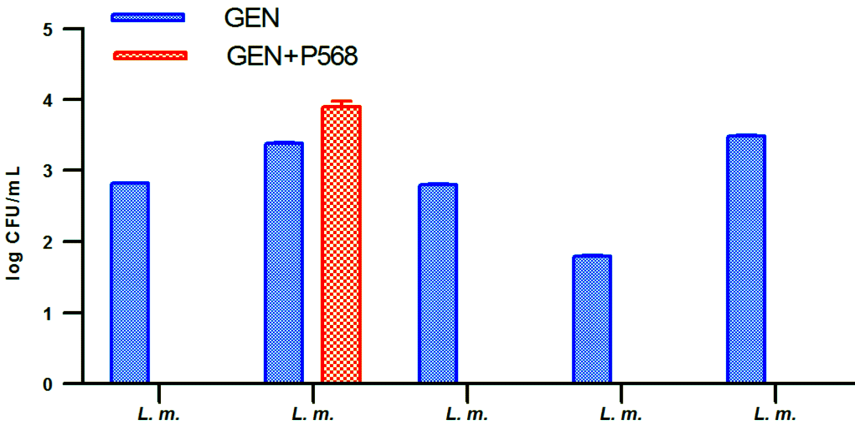

Figure 3 – *The graphic representation of the effectiveness of gentamicin incorporated in microcapsules of P. aeruginosa P568 polysaccharide versus gentamicin solution. L.m.: Listeria monocytogenes.*

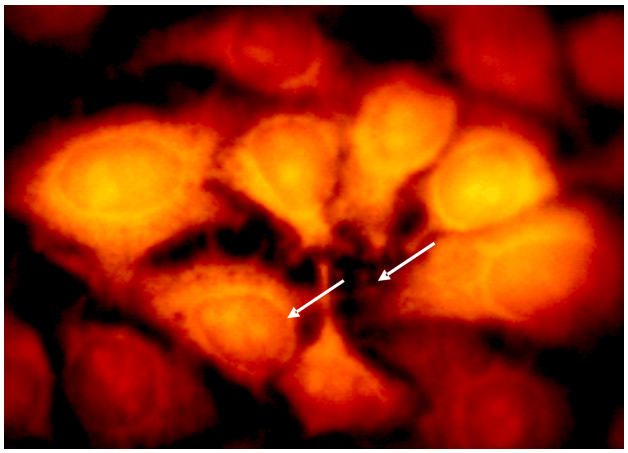

Figure 4 – *Propidium iodide (PI) fluorescence staining image representing HeLa cells in contact with the tested polysaccharides highlighting the fine cytoplasmic extensions and the presence of vacuoles in the cytoplasm (×1000).*

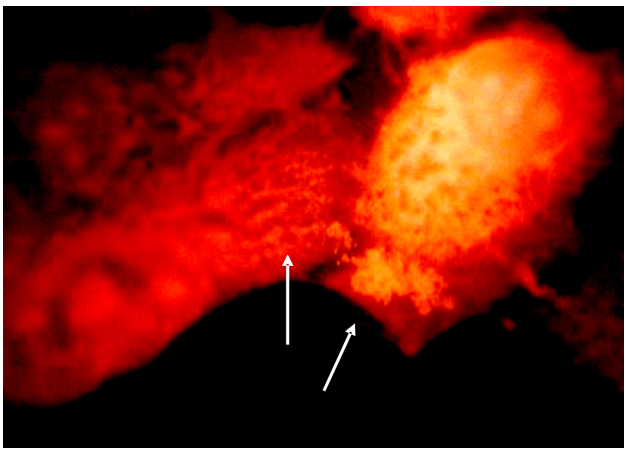

Figure 5 – *PI fluorescence staining image representing HeLa cells in contact with gentamicin loaded K800 polysaccharide outlining intracellular deposits of microcapsules (×1000).*

Discussion

Treatment of intracellular bacterial infections continues to be a medical challenge. Pathogens succeed to enter, survive, and some of them even multiply inside the mammalian cells, either phagocytic or non-phagocytic, cells where they are protected and escape from the humoral or cellular defense mechanisms of the host [24]. Internalized bacteria are also protected by antibiotic treatment because not all antibiotics could penetrate the cell. Being metabolically active, bacteria survive, and also could develop and spread antibiotic resistance [25]. It is absolutely necessary in these conditions to increase the efficiency of current antibiotics in the intracellular environment. Although a wide range of antibiotics are currently available, in fact many of them are not suitable for these pathogens because of their slow penetration, or their incapacity to exhibit optimal activity due to the physico-chemical conditions offered by the intracellular environments [26].

In this sense, the research studies are focused on finding some delivery systems for the delivery of the drugs directly into the target cells, eliminate the penetration problems on the one hand, and on the other hand to improve drug absorption, so thereby enable administration of lower doses and therefore limit adverse effects.

Until now, some natural and synthetic polymers proved to be efficient for encapsulating some antibiotics. Previous studies have shown that chitosan-coated plastic films, alone or loaded with antimicrobial agents, were effective against *L. monocytogenes* [27]. Liposomes containing DOPE (dioleylphosphatidylethanolamine) loaded with gentamicin proved to increase bactericidal activity against *L. monocytogenes* in mouse macrophages [27]. Other studies also found that gentamicin-loaded poly(D,L-lactide-*co*-glycolide) (PLGA) could be an alternative for the treatment of brucellosis, another intracellular infections [28, 29], this modality of aminoglycosides delivery being considered a better therapeutic approach in human brucellosis treatment for the past years [30].

Our results also seem to be promising, the microcapsules obtained from different bacterial species polysaccharides and loaded with gentamicin being efficient against the invasive bacterial cells belonging to different species. A possible explanation could be the stimulation of the endocytosis of the microcapsules loaded with antibiotic by the host cells, due tot he presence of the LPS, followed by the release of the antibiotic inside the cell and killing intracellular bacteria (Figure 6).

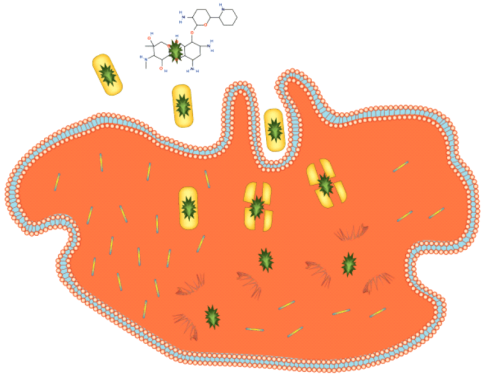

Figure 6 – ***Schematic representation of the proposed mechanism of intracellular delivery of gentamicin by the polymeric microcapsules.***

Conclusions

Both microscopy images and quantitative measurements of invasive bacterial cells demonstrate the internalization of the gentamicin-loaded polysaccharidic microcapsules and the effectiveness of the antibiotic against invasive intracellular bacteria. These results are promising and support the usefulness of these polysaccharidic compounds with bacterial origin for the design and optimization of new drug delivery systems for the controlled release of antibiotics in the intracellular environment.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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References

[1] Singh R, Lillard JW Jr. Nanoparticle-based targeted drug delivery. Exp Mol Pathol, 2009, 86(3):215–223.
[2] Pitt CG, Gratzl MM, Kimmel GL, Surles J, Schindler A. Aliphatic polyesters II. The degradation of poly (DL-lactide), poly (epsilon-caprolactone), and their copolymers *in vivo*. Biomaterials, 1981, 2(4):215–220.
[3] Pouton CW, Akhtar S. Biosynthetic polyhydroxyalkanoates and their potential in drug delivery. Adv Drug Deliv Rev, 1996, 18(2):133–162.
[4] Nobes GAR, Marchessault RH, Maysinger D. Polyhydroxyalkanoates: materials for drug delivery systems. Drug Deliv, 1985, 5(3):167–177.
[5] Zaki NM, Hafez MM. Enhanced antibacterial effect of ceftriaxone sodium-loaded chitosan nanoparticles against intracellular *Salmonella typhimurium*. AAPS PharmSciTech, 2012, 13(2):411–421.
[6] Rutten HJT, Nijhuis PHA. Prevention of wound infection in elective colorectal surgery by local application of a gentamicin-containing collagen sponge. Eur J Surg Suppl, 1997, 578:31–35.
[7] Vogelman B, Craig WA. Kinetics of antimicrobial activity. J Pediatr, 1986, 108(5 Pt 2):835–840.
[8] Sande MA, Mandell GL. Antimicrobial agents: the aminoglycosides. In: Goodman Gilman A, Rall TW, Nies AS, Taylor P (eds). Goodman and Gilman's the pharmacological basis of therapeutics. 8th edition, McGraw–Hill, New York, 1990.
[9] Gamazo C, Prior S, Concepción Lecároz M, Vitas AI, Campanero MA, Pérez G, Gonzalez D, Blanco-Prieto MJ. Biodegradable gentamicin delivery systems for parenteral use for the treatment of intracellular bacterial infections. Expert Opin Drug Deliv, 2007, 4(6):677–688.
[10] Nkabinde LA, Shoba-Zikhali LN, Semete-Makokotlela B, Kalombo L, Swai HS, Hayeshi R, Naicker B, Hillie TK, Hamman JH. Permeation of PLGA nanoparticles across different *in vitro* models. Curr Drug Deliv, 2012, 9(6):617–627.
[11] Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine, 2008, 42(2):145–151.
[12] Zhang Q, Liao GT, Wei DP, Zhang CJ. Increase of gentamicin uptake in cultured mouse peritoneal macrophage and rat hepatocytes when used in the form of nanoparticles. Yao Xue Xue Bao, 1996, 31(5):375–380.
[13] Francis L, Meng D, Knowles J, Keshavarz T, Boccaccini AR, Roy I. Controlled delivery of gentamicin using poly(3-hydroxybutyrate) microspheres. Int J Mol Sci, 2011, 12(7):4294–4314.
[14] Craig WA, Redington J, Ebert SC. Pharmacodynamics of amikacin *in vitro* and in mouse thigh and lung infections. J Antimicrob Chemother, 1991, 27(Suppl C):29–40.
[15] Dees C, Fountain MW, Taylor JR, Schultz RD. Enhanced intraphagocytic killing of *Brucella abortus* in bovine mononuclear cells by liposomes-containing gentamicin. Vet Immunol Immunopathol, 1985, 8(1–2):171–182.

[16] Prior S, Gander B, Lecároz C, Irache JM, Gamazo C. Gentamicin-loaded microspheres for reducing the intracellular *Brucella abortus* load in infected monocytes. J Antimicrob Chemother, 2004, 53(6):981–988.
[17] Maya S, Indulekha S, Sukhithasri V, Smitha KT, Nair SV, Jayakumar R, Biswas R. Efficacy of tetracycline encapsulated O-carboxymethyl chitosan nanoparticles against intracellular infections of *Staphylococcus aureus*. Int J Biol Macromol, 2012, 51(4):392–399.
[18] Yu J, Xie X, Zheng M, Yu L, Zhang L, Zhao J, Jiang D, Che X. Fabrication and characterization of nuclear localization signal-conjugated glycol chitosan micelles for improving the nuclear delivery of doxorubicin. Int J Nanomedicine, 2012, 7:5079–5090.
[19] Lozano MV, Esteban H, Brea J, Loza MI, Torres D, Alonso MJ. Intracellular delivery of docetaxel using freeze-dried polysaccharide nanocapsules. J Microencapsul, 2013, 30(2):181–188.
[20] Ojea-Jiménez I, Tort O, Lorenzo J, Puntes VF. Engineered nonviral nanocarriers for intracellular gene delivery applications. Biomed Mater, 2012, 7(5):054106.
[21] Cérantola S, Bounéry J, Segonds C, Marty N, Montrozier H. Exopolysaccharide production by mucoid and non-mucoid strains of *Burkholderia cepacia*. FEMS Microbiol Lett, 2000, 185(2):243–246.
[22] Croitoru C, Chifiriuc MC, Grumezescu AM, Lazar V. Optimization of culture conditions for the production and extraction of *Klebsiella* sp. and *Pseudomonas* sp. bacterial exopolysaccharides. Biointerface Res Appl Chem, 2013, 3(4):619–627.
[23] Cravioto A, Gross RJ, Scotland SM, Rowe B. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional enteropathogenic serotypes. Curr Microbiol, 1979, 3(2):95–99.
[24] Salouti M, Ahangari A. Chapter 5: Nanoparticle based drug delivery systems for treatment of infectious diseases. In: Sezer AD (ed). Application of nanotechnology in drug delivery. InTech, Rijeka, Croatia, 2014.
[25] Chifiriuc MC, Lixandru M, Iordache C, Bleotu C, Larion C, Dracea O, Lazar V, Antohe F, Israil AM. Internalization of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial cells by non-phagocytic, epithelial human cells. Rom Biotechnol Lett, 2008, 13(2):3651–3658.
[26] Carryn S, Chanteux H, Seral C, Mingeot-Leclercq MP, Van Bambeke F, Tulkens PM. Intracellular pharmacodynamics of antibiotics. Infect Dis Clin North Am, 2003, 17(3):615–634.
[27] Mu H, Guo F, Niu H, Liu Q, Wang S, Duan J. Chitosan improves anti-biofilm efficacy of gentamicin through facilitating antibiotic penetration. Int J Mol Sci, 2014, 15(12):22296–22308.
[28] Lecaroz C, Gamazo G, Blanco-Prieto MJ. Nanocarriers with gentamicin to treat intracellular pathogens. J Nanosci Nanotechnol, 2006, 6(9–10):3296–3302.
[29] Lecaroz C, Gamazo G, Renedo MJ, Blanco-Prieto MJ. Biodegradable micro- and nanoparticles as long-term delivery vehicles for gentamicin. J Microencapsul, 2006, 23(7):782–792.
[30] Van Bambeke F, Glupczynski Y, Plésiat P, Pechère JC, Tulkens PM. Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. J Antimicrob Chemother, 2003, 51(5):1055–1065.

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