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Amikacin loaded PLGA nanoparticles against *Pseudomonas aeruginosa*

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ABSTRACT

Amikacin is a very effective aminoglycoside antibiotic but according to its high toxicity, the use of this antibiotic has been limited. The aim of this study was to formulate and characterize amikacin loaded PLGA nanoparticles. Nanoparticles were synthetized using a solid-in-oil-in-water emulsion technique with different ratio of PLGA 50:50 (Resomer 502H) to drug (100:3.5, 80:3.5 and 60:3.5), two different concentrations of stabilizer (pluronic F68) (0.5% or 1%) and varied g forces to recover the final products. The most efficient formulation based on drug loading (26.0 ± 1.3 μg/mg nanoparticle) and encapsulation efficiency (76.8 ± 3.8%) was the one obtained with 100:3.5 PLGA:drug and 0.5% luronic F68, recovered by 20,000 × g for 20 min. Drug release kinetic study indicated that about 50% of the encapsulated drug was released during the first hour of incubation in phospahte buffer, pH 7.4, 37 °C, 120 rpm. Using different cell viability/cytotoxicity assays, the optimized formulation showed no toxicity against RAW macrophages after 2 and 24 h of exposure. Furthermore, released drug was active and maintained its bactericidal activity against *Pseudomonas aeruginosa* in vitro. These results support the effective utilization of the PLGA nanoparticle formulation for amikacin in further in vivo studies.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium which is extensively found in soil, water, plants and animals (Lang et al., 2004; Lyczak et al., 2000). It is one of the most important opportunistic pathogens and rarely causes disease in healthy persons (Abdi-Ali et al., 2006). However, the bacterium is easily able to infect immunocompromised and catheterized patients, patients with burn or traumatic wounds, malignancy, AIDS, cystic fibrosis (CF) and artificially ventilated individuals (Lang et al., 2004; Sabaeifard et al., 2014). Also, *P. aeruginosa* is considered as one of the main causes of nosocomial infections (Breidenstein et al., 2011; Chakraborty et al., 2012). In addition, increasing the morbidity and mortality associated infections, this species is highly resistant to a variety of antibiotics (Breidenstein et al., 2011; Chakraborty et al., 2012).

Aminoglycosides is one of the most effective family of antibiotics generally used in the treatment of Gram-negative infections (Ghaffari et al., 2011; Jana and Deb, 2006). Particularly, amikacin is an anti-pseudomonas antibiotic and the second drug of choice in cystic fibrosis centers. Also, it is the preferred antibiotic in treatment of nosocomial infections (Jana and Deb, 2006). This drug binds irreversibly to 30S ribosomal subunit and inhibits an initiation complex formation with mRNA for protein synthesis, thus, prevent protein synthesis and results in cell death (Ehsan et al., 2014; Ghaffari et al., 2011; Jana and Deb, 2006; López-Díez et al., 2005). Besides, as being cationic, aminoglycoside antibiotics also cause membrane damages and altered ionic concentration (Jana and Deb, 2006; López-Díez et al., 2005). However, due to their nephrotoxicity and ototoxicity, aminoglycosides are prescribed in limited and controlled doses (Abdollahi and Lotfipour, 2012; Ratjen et al., 2009; Zhang et al., 2010).

According to their sub-micron size, nanoparticles (NPs) are able to efficiently cross biological barriers (Parveen et al., 2012). In addition, improved drug bioavailability and resistance time in the body, protecting the drug from degradation and gradual drug release pattern are other advantages of nanoparticles (Mudshinge et al., 2011; Parveen et al., 2012; Zhang et al., 2010). These traits result in decrease in drug amount, dose related toxicity and side effects and therefore make nanoparticles proper candidates to deliver toxic drugs (Mudshinge et al., 2011). Recently, an inhaled liposome delivery system has been reported to be used to reduce the drug toxicity while increasing drug efficacy (Waters and Ratjen, 2014). Generally, the liposomal delivery systems are less stable in comparison to polymeric nanoparticles (Pinto-Alphandary et al., 2000). In this context, loading the drug in polymeric NPs could be of interest. To date, the only reported polymeric formulation loaded with amikacin has been synthetized by use of Eudragit®, while polymers like poly (D, L-lactideco-glycolide) (PLGA) in spite of their unique characteristics, as biodegradability and biocompatibility, have not yet been used in the development of amikacin-loaded nanoparticles (Sharma et al., 2015).

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In the present study, we investigated whether the bactericidal effect of amikacin against *P. aeruginosa* biofilms could be increased by encapsulating the antibiotic in a new formulation based on PLGA nanoparticles. Therefore, we synthetized amikacin-loaded nanoparticles (A-NPs) with different ratios of PLGA 50:50 (502H) to drug and different concentrations of pluronic F68. Drug release kinetic studies and cytotoxicity assays were performed to select the optimal formulation to perform the cytotoxicity and bactericidal activity in vitro studies. The results obtained support the effective utilization of the PLGA nanoparticle formulation for the treatment of persistent *Pseudomonas* biofilm infections.

2. Materials and methods

2.1. Materials

Amikacin hydrate was purchased by TOKU-E (Bellingham, USA). Poly (D, L-lactideco-glycolide) (PLGA) 50:50 (Resomer® RG 502H) was obtained from Boehringer-Ingelheim (Ingelheim, Germany). Fluoraldehyde™ O-phthalaldehyde Reagent Solution (OPA) was from Thermo scientific (Barcelona, Spain). 2-[*N*-morpholino] ethanesulfonic acid monohydrate (MES) and Pluronic® F-68 were from Sigma-Aldrich Co. (St. Louis, USA). Acetone was purchased by VWR (Barcelona, Spain). Trypticase soy both (TSB) was purchased by BioMérieux (Marcy l'Etoile, France). Mueller Hinton broth II (cation adjusted) was from BBL™ (Maryland, USA).

2.2. Nanoparticles preparation

2.2.1. Amikacin-loaded nanoparticles (A-NPs)

A-NPs were prepared by solid-in-oil-in-water method. Amikacin (3.5 mg) was dissolved in 100 μL ultrapure water. The drug solution was then added to 2 mL acetone containing varying amount of PLGA (60, 80 or 100 mg). The s/o phase was added to 10 mL pluronic F-68 (0.5% or 1% in 25 mM MES buffer) solution at pH 10. A-NPs were recovered by emulsions centrifugation at 20,000 × *g* for 20 min. Recovered A-NPs were freeze-dried in presence of 5% sucrose for 72 h.

2.2.2. Blank nanoparticles (NPs)

One hundred milligram PLGA was dissolved in 2 mL of acetone. One hundred microliters ultrapure water added to polymeric solution. Polymeric solution was then added to 10 mL pluronic F-68 (in 25 mM MES buffer) solution at pH 10, fully stirred at 300 rpm. NPs were recovered as described for A-NPs.

2.3. Nanoparticles characterization

2.3.1. Particle size and zeta potential

Particle size and zeta potential of the formulations were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetaplus apparatus (Brookhaven Instrument Corporation, USA).

The diameter of the particles was determined before and after lyophilization by dispersion of nanoparticles in ultrapure water. The measurements were done at 25 °C with a scattering angle of 90°.

Zeta potential was measured after addition of 1 mM pH 6 KCl solution to above nanoparticles solutions (Penalva et al., 2015).

2.3.2. Drug loading

To determine the amount of drug encapsulated in 1 mg of A-NPs, the amount equal to 5 mg of each formulation was dissolved in 1 mL of 1 M NaOH. The solutions were diluted 1:9 with 0.4 M boric acid pH 9.7. Fifty microliter of the diluted samples was added to 50 μL of OPA reagent. Fluorescence was measured immediately at a $\lambda_{ex}/\lambda_{em}$ of 340/450 (Tecan GENios fluorimeter (Tecan Group Ltd., Maennedorf, Switzerland)), respectively, and compared to a calibration curve of amikacin in 0.4 M boric acid pH 9.7 (Imbuluzqueta et al., 2011). Drug loading was calculated as the amount of drug (μg) per unit of mass of A-NPs (mg). The encapsulation efficiency was calculated as the ratio between the amount of entrapped drug and the initial amount of drug added and expressed in percentage.

2.3.3. Scanning electron microscopy (SEM)

A drop of freshly prepared A-NPs was placed onto a double-faced adhesive tape on a metal stub and dried overnight. The samples were coated by gold to a 16 nm thickness (Emitek K550) and observed by SEM (Zeiss DSM 940A, Germany).

2.3.4. Transmission electron microscopy (TEM)

Two milligram of the sample was diluted with ultrapure water and 10 μL of the suspension was placed on a 200-mesh Formwar-coated copper grid (EMS, FF200-Cu). The grid was left for 30 s at room temperature and washed 3 times with ultrapure water. Then, uranyl acetate (3%) was dropped onto the grids (5 min) to stain the sample. The images were captured using a 120 kV ZEISS Libra 120 electron microscope.

2.3.5. In vitro release profile

Release profile from A-NPs was studied using a dialysis technique. The amount equal to 10 mg of the A-NPs was dispersed in 1 mL phosphate buffered saline (PBS) pH 7.4 and placed in a dialysis bag (Spectra/Por, molecular weight cutoff 12,000–14,000 Da). To keep the sink condition, dialysis bag was placed in 24 mL PBS. Incubating at 37 °C under orbital shaking, at pre-determined intervals (0.5, 1, 2, 3, 4, 6, 8, 10 and 22 h), the whole receiver compartment was collected and replaced with the equal amount of fresh PBS (Abdelghany et al., 2012). The amount of released drug was determined as described in section 2.3.2.

2.4. Cell viability and toxicity assay

2.4.1. RAW 264.7 cell line culture

Cell viability assays were done using mouse macrophage cell line, RAW 264.7 (ATCC TIB-71). RAW macrophages were maintained in RPMI medium 1640, supplemented with 1% penicillin-streptomycin (10.000 U/mL, Gibco, CA, USA) and 10% fetal bovine serum (Gibco, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Briefly, 10^5 cells per well cells were seeded in 96-well plates and incubated for 24 h. Then, 100 μL of freshly prepared nanoparticles suspension and free drug solution in respective medium (0.01–1 mg/mL) were replaced with the old culture medium. Two sets of controls treated with culture medium (live control) and cells treated with Triton™ X-100 1% (*w*/*v*) in culture medium (dead control), respectively, were also included. After the incubation time, the culture medium was removed and the cells were washed twice with PBS. Cell viability assays were done after 2 and 24 h of exposure to formulations using the 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide (MTT) test (Mosmann, 1983), alamar blue (AB) test and the neutral red uptake (NRU) test (Borenfreund and Puerner, 1985). Cell toxicity was assayed by use of Lactate dehydrogenase (LDH). Cell viability and cytotoxicity were assessed as a percentage in relation to controls.

Each experiment was repeated six times from three independent incubation preparations.

2.4.2. MTT cell viability assay

MTT assay depends on the mitochondrial reductive capacity to metabolize the MTT salt to a colored formazan product. The assay was done by adding 100 μL of MTT (0.5 mg/mL in RPMI) to each well. The plate was incubated for 4 h at 37 °C, 5% CO_2 in the dark. The MTT solution was discarded and formazan crystals were solubilized using 125 μL of dimethyl sulfoxide (Mosmann, 1983). The plate was shaken for 10 min at room temperature, and absorbance was measured at 540 nm using a microplate reader (Agilent, USA).