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ORIGINAL PAPER

Initial Development and Characterization of PLGA Nanospheres Containing Ropivacaine

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Abstract Local anesthetics are able to induce pain relief by binding to the sodium channels of excitable membranes, blocking the influx of sodium ions and the propagation of the nervous impulse. Ropivacaine (RVC) is an amino amide, enantiomerically pure, local anesthetic largely used in surgical procedures, which present physico-chemical and therapeutic properties similar to those of bupivacaine but decreased toxicity and motor blockade. The present work focuses on the preparation and characterization of nanospheres containing RVC; 0.25% and 0.50% RVC were incorporated in poly(*d*,*l*-lactide-*co*-glycolide (PLGA) 50:50) nanospheres (PLGA-NS), prepared by the nanoprecipitation method. Characterization of the nanospheres was conducted through the measurement of pH, particle size, and zeta potential. The pH of the nanoparticle system with RVC was 6.58. The average diameters of the RVC-containing nanospheres was 162.7 ± 1.5 nm, and their zeta potentials were negative, with values of about -10.81 ± 1.16 mV, which promoted good stabilization of the particles in solution. The cytotoxicity experiments show that RVC-loaded PLGA-NS generate a less toxic formulation as compared with plain RVC. Since this polymer drug-delivery system can effectively generate an even less toxic RVC formulation, this study is fundamental due to its characterization of a potentially novel pharmaceutical form for the treatment of pain with RVC.

Keywords Ropivacaine · Nanospheres · PLGA · Drug delivery

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1 Introduction

In spite of the recent advances in basic and clinical investigation of new therapeutic agents, the management of pain is still a challenge. Local anesthetics (LA) are among the different classes of pharmacological compounds used to attenuate or to eliminate pain. These drugs, which are able to reversibly block the excitation–transmission process in axons, have a relatively short action and a significant toxicity to the central nervous and cardiovascular systems [1].

Ropivacaine (RVC, Fig. 1), a new amide-type local anesthetic, used as the *S*-(−) enantiomer, is an *n*-propyl homolog of bupivacaine. Because of their similar pharmacodynamic properties, RVC and bupivacaine are used in a variety of clinical procedures [2]. They present a comparable clinical efficacy when compared as to latency, potency, and duration of action. However, the main features attributed to this new LA are that RVC provides a lower cardiac toxicity and allows an earlier motor blockade recovery in relation to bupivacaine [3–5]. Nowadays, there is a strong clinical requirement for controlled-release local anesthetics, as well as for LA molecules with low systemic uptake that could lead to less toxic side effects [6, 7].

Colloidal polymeric nanoparticles used as drug carriers can be made of artificial and natural polymers which must be biocompatible. To form the core of the carriers, different biodegradable materials are used. Nanoparticles, as a generic term, refers to nanospheres (NS) and nanocapsules, which are polymeric nanocarriers presenting matricial and vesicular structures, respectively [8]. Drug release from nanoparticulated systems depends on desorption, diffusion, particle erosion, or a combination of these factors [9].

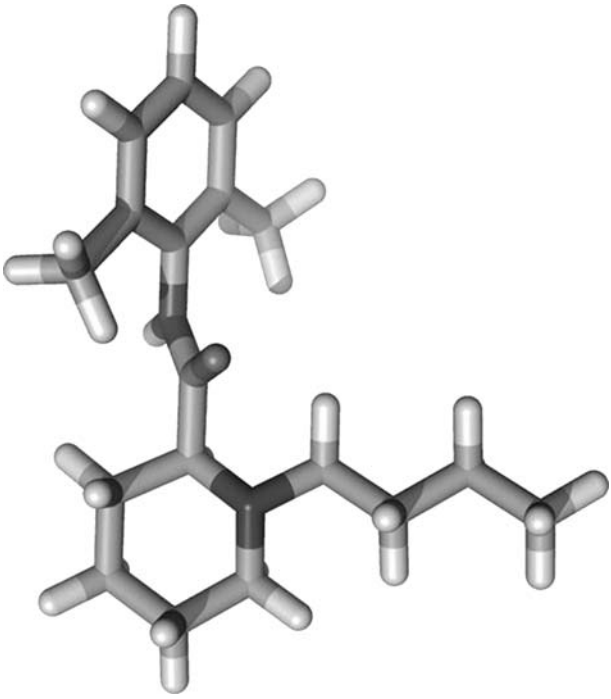

Fig. 1 Chemical structure of Ropivacaine

Biodegradable aliphatic polyesters of hydroxyl acids such as Poly(lactic-*co*-glycolic acid) (PLGA) are approved for use in humans by the Food and Drug Administration and have been extensively used in medicine in a variety of applications [10, 11]. PLGA undergoes no enzymatic hydrolysis to lactic and glycolic acids, which are eventually metabolized to carbon dioxide and water; so, this polymer is often considered a standard for drug delivery purposes.

In this work, the objective was to prepare and characterize a drug-delivery system that can effectively generate an even less toxic RVC formulation. This study is fundamental in its characterization of a potentially novel pharmaceutical form for the treatment of pain with RVC, a newer and safer long-acting local anesthetic than bupivacaine.

2 Experimental

2.1 Reagents and Chemicals

Poly(*d*,*l*-lactic-*co*-glycolic) (MW 60000) and polyvinyl alcohol (PVA) were supplied by Sigma Chemical Company. RVC was kindly given by Cristália (Itapira, Brazil). All other chemicals were reagent grade.

2.2 Preparation of Nanospheres

The preparation of empty and RVC-loaded PLGA-NS was based on a solvent-displacement process [12]. Briefly, 60 mg of PLGA and 0.25% or 0.50% of RVC were first dissolved in 25 mL of acetone. This organic phase was poured into 30 mL phosphate buffer (pH 7.4, 5 mM) containing 100 mg PVA as the hydrophilic surfactant under moderate magnetic stirring. Finally, the organic solvents were evaporated under reduced pressure at 58°C, and the final volume of the aqueous suspension was adjusted to 10 mL.

2.3 Physicochemical Characterization of the Suspensions

2.3.1 Particle Size and Zeta Potential

The mean diameter of nanospheres in the dispersion was determined by photon correlation spectroscopy using a laser light scattering instrument (ZetaPlus, Brookhaven) at a fixed angle of 90° at 25°C. The particle size analysis data was evaluated using the volume distribution. Zeta potential was carried out using Zeta potential analyzer (Zeta plus, Brookhaven, NY, USA) at the same temperature. All preparations were diluted at 1/20 with deionized water and measured in triplicate.

2.3.2 pH

pH values of the nanoparticles (with or without RVC) aqueous suspensions were measured with a pH meter (Orion® pHmeter) by simply plunging the electrode into the nanosuspensions (with or without RVC).

2.4 Drug Entrapment Efficiency

Free RVC (non-associated with the nanostructures) was determined in the ultrafiltrate after separation of the nanoparticles by ultrafiltration or centrifugation technique (Ultrafree-MC 30.000 MW, Millipore). Total RVC concentration was measured using high performance liquid chromatography (HPLC) after dissolution of the colloidal dispersions by acetonitrile. The HPLC system consisted of a mobile phase delivery pump (Shimadzu LC—10AD), a UV-Vis detector (SPD—10A), and a 20−μL loop (Rhenodyne model). A C_{18} reverse-phase column (Hypersil ODS C_{18}, 5 μ 110A, 150 × 4,60 mm) and a Phenomenex C_{18} security guard were utilized for drug separation, using acetonitrile or phosphate buffer pH 7.4, 5 mM (95/5, *v*/*v*) as mobile phase. The flow rate and UV wavelength were 1.0 mL/min and 220 nm, respectively (analytical curve = Peak area = 27146.5[RVC] + 116734.1, $r = 0.9993$).

The entrapment efficiency (EE, %) of RVC associated with PLGA-NS was calculated from the difference between the total and the free drug concentrations, measured in the dispersions and in the ultrafiltrate, respectively (Eq. 1):

$$EE(\%) = \frac{W_s}{W_{\text{total}}} \times 100\% \tag{1}$$

where W_s: amount of RVC in PLGA-NS; W_{total}: amount of RVC used in formulation.

2.5 Cell Culture and Cytotoxic Assays

Balb/c mouse fibroblasts (3T3 cells) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 UI/mL penicillin, and 100 μg/mL streptomycin sulfate (pH 7.2–7.4) under a humidified atmosphere, at 37°C and 5% CO_2. Cells were seeded (2×10^4 cells/well) in 48-well tissue culture plates and cultured for 48 h. The cells were then incubated for 24 h with the test compound (RVC, PLGA-NS, or RVC-loaded PLGA-NS) at two different concentrations 0.25 and 0.5 mg/mL. Cell viability was assessed by tetrazolium reduction (MTT test). 1 mg/mL MTT was incubated for 1 h with the treated 3T3 cells at 37°C. The number of viable cells was determined by measuring the amount of MTT converted to insoluble formazan dye by mitochondrial dehydrogenases. The formazan crystals formed were dissolved in a 1 M HCl-isopropyl alcohol mixture (1:24 *v*/*v*) and shaken for 20 min at room temperature. Cytotoxic assay data were analyzed by one-way analysis of variance (ANOVA), with Tukey–Kramer as a post hoc test. Statistical significance was defined as $p < 0.001$.

3 Results and Discussion

3.1 Physicochemical Characterization of the Suspensions

The variation of the size of nanoparticles, zeta potential, and pH for RVC (0.5%)-loaded PLGA-NS incubated at 25°C for 30 days was shown in Table 1.

The initial values of size distribution and polydispersity were bellow 200 nm, in agreement with the characteristics of the colloidal dispersions obtained by the nanoprecipitation technique using PLGA as polymer [13]. The zeta potential presented negative values around

Table 1 Physico-chemical characteristics of PLGA-NP loaded with RVC (0.50%) as function of time; storage at 25°C, phosphate buffer (5 mM)

Time (days)	Particle size±S.D. (nm; polydispersivity)	Zeta Potential ± SD (mV)	pH
1	162.7 ± 1.5 (0.097 ± 0.023)	−10.81 ± 1.16	6.58 ± 0.21
15	162.3 ± 1.1 (0.062 ± 0.019)	−11.05 ± 1.42	6.38 ± 0.15
30	163.1 ± 2.1 (0.098 ± 0.033)	−9.36 ± 2.33	6.36 ± 0.16

−10 mV, enough to assure the physical stability of the systems [14]. The size of the PLGA-NS did not change significantly with time during the incubation period studied, and the small changes of pH values could indicate that no hydrolysis of PLGA polymer chains occurs [15–17].

3.2 Entrapment Efficiency

The technique for determining drug association with colloidal systems, ultrafiltration or centrifugation, was used for determination of the EE (%) for RVC in PLGA-NS. The free RVC and loaded RVC in PLGA-NS determined from the ultrafiltration method were 96.2% and 3.8%, respectively. The result shows that RVC has a low entrapment efficiency (3.8%) in PLGA-NS; this is probably because of the physico-chemical characteristics of the RVC molecule, such as water solubility.

In this case, RVC molecules (pKa about 8.0) were presented in a charged form (with good water solubility, 30 mM) because in PLGA-NS suspensions, the pH was about 6.5. It has been shown that PLGA-NS presents low drug incorporation efficiencies, especially of water soluble drugs, due to their small size and, hence, large surface area, which promotes drug loss into the aqueous phase during particle formation [18]. Similar results were found for procaine (a local anesthetic) in PLA–PEG copolymers, with an EE (%) of about 6% [18]. Another important point is that the lower association of RVC in PLGA-NS can be due to the method of nanosphere preparation. It has been suggested that a w/o/w emulsification solvent evaporation method of nanosphere preparation might result in higher drug loading [9].

3.3 Cell Culture and Cytotoxic Assays

Measurement of the effect of RVC, PLGA nanospheres, and RVC-loaded PLGA nanospheres on the 3T3 cell viability is a way to evaluate the cytotoxicity of these chemical substances. The 3T3 cells were treated at two different concentrations (0.25% and 0.50%) of each compound. Figure 2 shows the effect of concentration on the 3T3 cell viability (%), with significant differences between the results for RVC and RVC-loaded PLGA nanospheres.

Varying the concentration of PLGA-NS had no effect on cell viability. On the other hand, RVC reduced cell viability in a dose-dependent manner, down to 40% (0.50%), while RVC-loaded PLGA-NS induced a maximum inhibition (0.50%) comparable to that of PLGA-NS, i.e., affecting cell viability very little up to 24 h after treatment ($p < 0.001$), compared to RVC (Fig. 2). One of the essential points about this in vitro toxicity model is that, since the cytotoxic effects of RVC are dose-dependent, the cellular protective effects observed on treatment with the RVC-loaded PLGA-NS could be explained by the sustained release of RVC from the nanospheres.

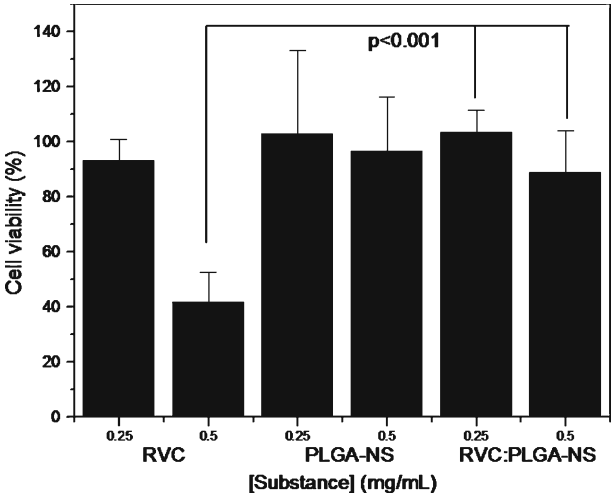

Fig. 2 Cytotoxic effects of RVC, PLGA-NS, PLGA-NS:RVC at 0.25 and 0.5 mg/mL on Balb/c 3T3 cells incubated for 24 h at 37°C and 5% CO_2 as evaluated by MTT reduction test. Data expressed as percent cell viability (Mean ± SD, $n = 8$ experiments). $p < 0.001$ (one-way ANOVA with Tukey–Kramer post hoc test)

4 Conclusions

The results showed that RVC-loaded PLGA-NS were stable after 30 days and decrease the cytotoxicity of RVC compared to RVC alone. This formulation has been investigated for its potential use as a new therapeutic formulation, to decrease the systemic toxicity of RVC. This study provides new perspectives for future experiments using these nanoparticles of PLGA with local anesthetics in order to verify their therapeutic efficacy.

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