RESEARCH ARTICLE

Arch Pharm Res Vol 31, No 11, 1509-1516, 2008
DOI 10.1007/s12272-001-2137-7

Archives of Pharmacal Research
www.springer.com/12272

Microencapsulation of Antibiotic Rifampicin in Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

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(Received July 24, 2008/Revised October 16, 2008/Accepted October 16, 2008)

The aim of this study was the preparation of microparticles containing rifampicin using a biodegradable polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) for oral administration produced by a bacteria. The poly(3-hydroxybutyrate-co-3-hydroxyvalerate) microparticles with and without rifampicin were prepared by the emulsification and solvent evaporation method, in which chloroform and polyvinyl alcohol are used as the solvent and emulsifier, respectively. Microparticles were obtained within a size range of 20-60 μm by changing the initial poly(3-hydroxybutyrate-co-3-hydroxyvalerate), polyvinyl alcohol and rifampicin concentrations. An encapsulation efficiency value of 14% was obtained. The optimized total yield of 60% of the poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/ rifampicin was obtained. A load of 0.035 mg/1 mg of PHBV was reached. Almost 90% of the drug loaded in the microparticles was released after 24 h. The size, encapsulation efficiency and ribampicin release of the microparticles varied as a function of the initial poly(3-hydroxybutyrate-co-3-hydroxyvalerate), polyvinyl alcohol and rifampicin concentrations. It was demonstrated that the microencapsulated rifampicin, although was not totally available in the medium, exhibited a similar inhibition value as free rifampicin at 24 h of incubation with *S. aureus*. Cytotoxicity assays demonstrated a reduction of the toxicity when rifampicin was microencapsulated in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) while maintaining its antibacterial activity.

Key words: Controlled release/delivery, Emulsion/microemulsion, Biomaterial, Microparticles, Biodegradable polymers, Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), Rifampicin, Antibacterial Activity, Cytotoxicity

INTRODUCTION

Staphylococcus aureus (coagulase-positive species) is an important nosocomial pathogen that causes skin infections, bacteraemia, pneumonia, osteomyelitis, endocarditis, myocarditis, meningitis and abscesses at different sites. One of the major clinical and also epedimiological problem in hospitals is Methicillinresistant *S. aureus* (MRSA). Bacteraemia related to foreign bodies and indwelling medical devices are usually related to *S. aureus* and its relative *Staphylococcus epidermidis*. On the inert surfaces of devices, the bacteria are able to grow as biofilms, resistant to antimicrobial agents. Unfortunately, the removal of the infected device is often the only possible clinical solution (Petrelli et al., 2008). Different antibiotics are being used in these kind of infections and one of the common treatment is with antibiotics such as rifampicin (RIF). However, many secondary effects were observed with long treatment with RIF such as asymptomatic jaundice, muscular and articulate pain, etc (Campbel et al., 2001).

A therapeutic technique has been developed to increase the drug concentration at the site of infection and/or decrease the toxicity of the drug and/or maintenance of drug levels within a desired range (Marcato and Durán,

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2008). This technique is drug encapsulation in liposomes (Kamath et al., 2000; Labana et al., 2002) or in micro- and nanoparticles of different polymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Durán et al., 2007; Schaffazick et al., 2003; Chen and Davies, 2002).

RIF alone or associated with another drug was encapsulated in different nanostructures and especially in nanoparticles. Promising results were obtained by Zahoor et al. (2005) in the study with RIF encapsulated in alginate nanoparticles. They observed that the chemotherapeutic efficacy of three doses of drug-loaded alginate nanoparticles nebulized 15 days apart was comparable with 45 daily doses of oral free drugs. Studies of tuberculosis in mice using nanocapsules of poly(n-butylcyanoacrylate) and poly(butylcyano acrylate) containing isoniazid, RIF and streptomycin which were injected in only one dose resulted in a controlled release to several mouse organs decreasing the CFU at 8 days of infection (Anisomov et al., 2000). RIF, isoniazid and pyrazinamide encapsulated in poly (DL-lactide-co-glycolide) (PLGA) nanoparticles suitable for nebulization was shown to be a sound basis for improving drug bioavailability and reducing the dosing frequency for better management of pulmonary tuberculosis (Esmaili et al., 2007). The effect of nanoencapsulation of RIF in PLGA on the antibacterial activity of RIF against gram-positive and gram-negative bacteria was evaluated and it was shown that RIF nanoparticles could considerably improve the RIF antibacterial efficacy (Pandey et al., 2003; Esmaili et al*.,* 2007). Similar effect for isoniazid encapsulation in poly(ε-caprolactone) (PCL) was observed (Durán et al., 2006). A study with a murine tuberculosis model using oral PLGA nanoparticle with encapsulated ethambutol in combination with RIF, isoniazid and pyrazinamide showed that the polymeric nanoparticle based oral four-drug combination has significant potential to shorten the duration of tuberculosis chemotherapy as well to reduce the dosing frequency (Pandey et al., 2006a; Pandey et al., 2006b). In *M. tuberculosis* H37Rv infected mice, no tubercle bacilli could be detected in the lungs/spleen after 5 oral doses of a the drug encapsulated in solid lipid nanoparticles administered every ten day; whereas 46 daily doses of oral free drugs were required to obtain an equivalent therapeutic benefit (Pandey et al., 2005). Alginate nanoparticle encapsulated econazole and antitubercular drugs against murine tuberculosis were studied and econazole (free or encapsulated) could replace RIF and isoniazid during chemotherapy of murine tuberculosis (Zahoor et al., 2007).

Few reports on microparticles with RIF encapsulation have been published (Tomoda et al., 2005; Tomoda and Makin, 2007). Encapsulation of RIF in inhalable poly (lactide) (PLA) microparticles containing two anti-tuberculosis agents, isoniazid and RIF, efficiently induced tumour necrosis factor-α (TNF-α) secretion by macrophages recovered from infected mice (Sharma et al., 2007; Harma et al., 2001). Alginate microparticles containing antitubercular drugs were evaluated *in vitro* and *in vivo* release profiles. These microparticles exhibited sustained release of isoniazid, RIF and pyrazinamide for 3-5 days in plasma and up to 9 days in organs (Ul-Ain et al., 2003). Very recently, lipid microspheres containing RIF were showed to deliver the drug to alveolar macrophages *in vitro* and *in vivo*, and that intranasal administration to animals could achieve preferential accumulation in the lungs with less effect on the liver (Takenaga et al., 2008).

RIF has also been encapsulated in poly(hydroxybutyrate) (PHB) microspheres by solvent evaporation (particle sizes 5-100 μm) and an the efficacy of controlled RIF release was demonstrated (Kassab et al., 1997). PLGA microspheres containing RIF or isoniazid have been succefully prepared for the direct delivery of these drugs to the lung, but unfortunately, the cost of this polymer is too high (Fumori and Makino, 2004).

PHB and PHBV are novel biodegradable and biocompatible polymers with lower cost that are starting to draw the attention of scientist working in several fields of science, including medicine, pharmacy, cosmetics and agriculture. These polymers are ideal for use as biomedical materials due to their unique and interesting physicochemical features, in addition to their mechanical properties which are similar to those of PLGA (Sendil et al*.,* 1999) and therir non-toxic nature (Dang et al., 1999).

Moreover, PHBV does not induced acute inflammation, abscess formation or tissue necrosis in tissues with PHBV implant (Gogolewski et al*.,* 1993). These attractive features make PHBV an attractive material for the drug delivery systems (Pich et al*.,* 2006).

The aims of this study were to use PHBV as coating material in microparticles prepared with and without RIF for sustained liberation systems and to analyze and the properties of the resultant systems *in vitro*. The morphological changes before and after release was assessed by Scanning Electron Microscopy (SEM). Also, bioassays using a RIF-sensitive organism such as *Staphylococcus aureus* and cytotoxicity studies in Chinese fibroblast V79 cells were carried out.

MATERIALS AND METHODS

Materials

PHBV (PHB-9.8%HV) (MW 92 kDa) was kindly provided by Biocycle (São Paulo, Brazil); Polyvinyl alcohol (PVA) (MM 30-70 kDa) was purchased from Aldrich (St. Louis, USA); RIF and Chloroform were obtained from Sochimia Srl-Grupo Lepetit Commercial Business (Milan,

Italy) and Synth (São Paulo, Brazil), respectively.

Preparation of RIF microparticles

The microparticles were prepared by a slightly modified oil/water (o/w) emulsification method proposed by Kassab et al. (1997): First, an excipient solution was prepared by dissolving approximately 0.05-0.20 g of PHB-9.8%HV in 5 mL chloroform (PHBV was insoluble in most common organic solvents). To this solution, approximately 15-50 mg of RIF was added by thorough mixing and a homogeneous solution was obtained. The aqueous phase was prepared in 100 mL of water (Milli-Q) with 1.0-1.2% PVA with mechanical stirring at 2400 rpm for 20 min. After the organic phase was added dropwise to the aqueous phase under mechanical stirring at 1000 rpm (~30 min), the organic solvent was evaporated for 12 h at 25ºC. The microparticles were colleted by filtration using a 0.22 μm nylon filter and then the particles on the filter were washed with Milli-Q water and freeze-dried.

RIF content in the microparticles

The RIF content in the microparticles was measured by dissolving 10 mg of microparticles in 1.0 mL dichloromethane and 9 mL of methanol. The suspension was centrifuged at 4000 rpm for 5 min and the supernatant was analyzed by a spectrophotometer (Agilent 8453-diode array) ($\lambda = 334$ nm) using an analytical curve previously construction with RIF dissolved in dichloromethane/methanol (10% v/v) (Kassab et al., 1997). The total amount of RIF in microparticles was determined in duplicate and expressed as encapsulation efficiency (EE), drug content and drug yield as previously described in the literature (Govender et al., 1999).

***In vitro* release analysis of encapsulated RIF**

In order to assess the release kinetics of RIF from the microparticles, 5 mg of microparticles (from at least two different batches) were weighed into glass test tubes (16 × 100 mm). To each tube, 10 mL of 50 mM phosphate buffer (PBS) at pH 7.4 with 10% of ethanol was added. The test tubes were placed in an incubator maintained at 36.5 ± 0.1ºC under gentle agitation at 120 rpm. After 1, 3, 6, 11 and 23 h, 1 mL was removed and replaced with new PBS buffer. Concentrations of RIF were determined spectrophotometrically (Agilent 8453 – diode array) ($\lambda =$ 473 nm). The same experiment was carried out with RIF free nanoparticles.

Characterization of microparticles by scanning electron microscopy (SEM)

The SEM image was used to study the shape and surface morphology of the particles. Previously, the samples were coated with 10 nm of gold/palladium under vacuum by sputtering using a BAL-TEC's apparatus and characterized by SEM at a voltage of 20 kV (Jeol - JSM-6360LV). Secondary electron images were obtained. The approximately sizes of the particles was measured by SEM image of the microparticles using the software Image Tool (UTHSCSA 3.0) with n=20. Measurements by LS Particle Size Analizer/230 (Beckman Couter) were also carried out.

***In vitro* test of antibacterial activity**

The antibacterial activity test (AAT) was carried out in triplicate by the Agar Diffusion Method (Borin et al., 2007). Briefly, 30 mL of PCA (Plate Count Agar) were added to each Petri dish and then inoculated with 100 μL of bacteria solution that contained approximately 10^5 CFU/mL of *Staphylococcus aureus* (ATCC 6538). After the gelification of Agar, a cavity of 6 mm of diameter in the central region of the plate was formed. In the cavities of each plate, 100 mL RIF solution as control (4 mg/mL) or 100 mL PHBV microparticles with RIF (4 mg/mL) or 100 mL PHBV microparticles without RIF were added. After 24 h of incubation at 37ºC, bacterial growth inhibition areas around wells were measured in order to evaluate antibacterial activities of the samples.

Cytotoxicity assay

V79 fibroblast culture: The cytotoxicity of RIF and its microparticles was assessed in a permanent lung fibroblast cell line derived from chinese hamsters (V79) (Corrêa et al. 2005). V79 fibroblasts were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37ºC. The cells were plated at a density of 3×10^4 cells/mL in 96-well plates. The medium was removed 48 h after cell seeding and replaced with medium containing RIF at several concentrations.

Cells treatment

Free RIF and RIF encapsulated in PHBV microparticles were initially dissolved in DMEM with dimethyl sulfoxide (DMSO) at concentrations from 0 to 1000 μM for free RIF and at a concentration of 400 μM for microencapsulated RIF. The final concentration of DMSO in the test medium and controls was 0.2%. The cells were exposed for 24 h to the test medium with or without the compounds studied (control). Each concentration was tested in six replicates in each of three separate experiments. At the end of the incubation, three independent endpoints for cytotoxicity were evaluated: nucleic acid content (NAC), reduction of a tetrazolim bromide (MTT) and neutral red uptake (NRU).

Endpoint tests for cytotoxicity

Nucleic acid content (NAC)

The number of cells in the control and treated wells was estimated from the total nucleic acid content (Cingi et al., 1991). After treatment, the cells were washed once with cold phosphate-buffered saline (PBS). The cell monolayers were then digested in 0.5 M NaOH (0.1 mL/well) at 37°C for 1 h. The absorbance of the NaOH fraction, measured at 260 nm (UV-visible DU® 640B Spectrophotometer, Beckman Instruments, Inc., Fullerton, CA, USA), was used as an index of cell number and the results were expressed as a percentage of the 260 nm absorbance of the control wells (Bianchi and Fortunati, 1990).

Methylthiazoletetrazolium reduction (MTT)

The MTT reduction assay was carried out as described by Denizot and Lang (1986). Briefly, cells were washed once with PBS before adding 0.1 mL of serum-free medium containing MTT salt (1 mg/mL) to each well. After incubation for 4 h, the culture medium was removed and 0.1 mL of ethanol was added to each well to solubilize the formazan formed. The plates were shaken gently for 10 min on a microtitre plate shaker and the absorbance was measured at 570 nm (VersaMax™, Tunable Microplate Reader, Molecular Devices, Co., Sunnyvale, CA, USA).

Neutral red uptake (NRU)

The neutral red uptake was measured by the method of Borefreund and Puerner (1984). Briefly, cells were washed once with PBS after removal of the culture medium and 0.1 mL serum-free medium containing neutral red (50 μg/mL) was added to each well. After 4 h of incubation, the cells were washed with PBS-Ca^{2+} and then 0.1 mL of a solution of 1% acetic acid (v/v): 49% ethanol (v/v) was added to each well to fix the cells and to remove the neutral red from the solution. The plates were then shaken gently for 10 min on a plate shaker and the absorbance of the solution was read at 540 nm (VersaMax™, Tunable Microplate Reader Molecular Devices, Co., Sunnyvale, CA, USA).

Statistical analysis

The experiments were repeated three times (six replicates each) in separate experiments. To calculate the IC_{50} values (concentration that produced a 50% inhibitory effect on the evaluated parameter), the results were expressed as a percentage of the controls and were determined graphically from the concentration–response curves using the computer software package Origin®-Data Analysis and Technical Graphics, version 6.0 (Copyright Software, Inc.).

RESULTS AND DISCUSSION

The PHBV microparticles with and without RIF, which were prepared by the emulsification/evaporation technique followed by solvent evaporation as previously described, are spherical, porous and with a rough surface texture as show Fig. 1.

PHBV microparticles were prepared with different initial concentrations of RIF, PVA and PHBV in order to study the influences of these parameters on the microparticle morphology, EE and sustained drug release (Table I).

The EE and the RIF content varied significantly with the PHBV/solvent ratio, as shown in Table I (runs 2 and 4). Higher PHBV concentration (10 mg/mL to 40 mg/mL) increases the EE from 2.8% to 13.9% and RIF content from 2.5% to 4.8%, indicating the influence of polymer concentration on RIF encapsulation. Moreover, the increase of the PHBV concentration resulted in an increase in the microparticles size from 29 for 43 μm (Table I). However; the morphology of these particles did not change as shown in the SEM micrographs in Fig. 2. These results can be explained by the variation in the organic phase viscosity. The high amount of the polymer increases the drop's viscosity and consequently decreases the speed of mass transfer contributing to a high drug encapsulation. Furthermore, the increase of viscosity

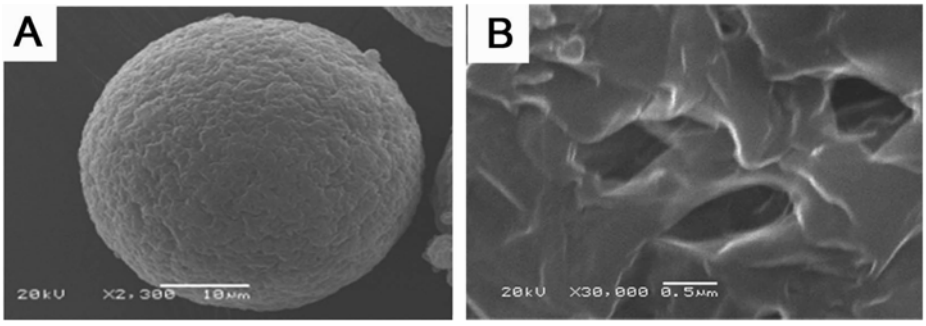

Fig. 1. SEM micrographs of PHBV microparticles (Run 4) (see Table I): A) 2300×, B) 30000×

Table I. Concentration effect of RIF, PHBV and PVA in the EE, RIF content, yield, microparticles sizes and drug release

Runs	[PHBV] (mg/mL)	[RIF] (mg/mL)	[PVA] (%)	EE[a] (%)	RIF[a] Content (%)	Yield[a] (%)	Size (mumum) *[b]	Drug release in 24 h[a] (%)
1	10.0	10.0	1.2	0.7±0.01	0.9±0.05	38±0.4	25±1.2	99±4
2	10.0	10.0	1.0	2.8±0.11	2.5±0.01	50±0.5	29±1.4	100±4
3	40.0	3.9	1.0	8.2±0.24	2.5±0.10	34±0.3	40±2.0	94±3
4	40.0	10.0	1.0	13.9±0.27	4.8±0.24	60±0.6	43±2.1	93±4

* measured by SEM.
[a] Values are expressed as mean SD (n = 2)
[b] Values are expressed as mean SD (n = 20)

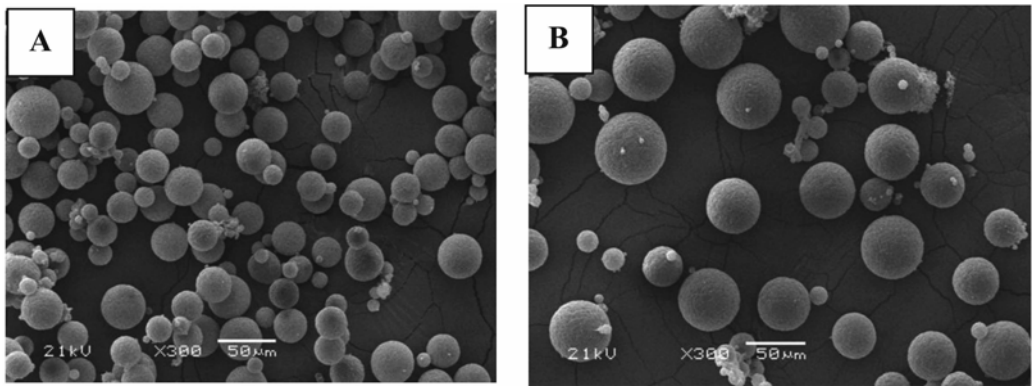

Fig. 2. SEM micrographs of PHBV microparticles with RIF prepared with A) (Run 2, Table I) (300×); B) (Run 4. Table I) (300×)

complicates the microparticles dispersion in the aqueous medium and increases its sizes. A similar behavior was found using the polymers PHB and polyethyleneglycol/ polylactide (Kassab et al., 1997; Celikkaya et al., 1996). In the case of RIF in PLGA, the values of encapsulation were over 40% (Tomoda and Makino, 2007) but the charge ratio in this case was 0.10 mg RIF/1 mg of polymer (loading of RIF of 0.036 mg/1 mg PLGA). In contrast, with PHBV was 0.25 mg RIF/1 mg of polymer (See Table I) (loading of RIF of 0.035 mg/1 mg PHBV) was obtained which is a 2.5 fold higher RIF concentration in PHBV than PLGA with the same amount of drug loading.

Another parameter that can influence the EE of RIF is the PVA concentration (Table I, runs 1 and 2). The same concentration of RIF and PHBV (10 mg/mL) and different PVA concentrations (1.2% and 1.0%), resulted in EE of 0.7% and 2.8%, respectively, and RIF content of 0.9% and 2.5%, respectively. These results showed that a low PVA concentration favored RIF encapsulation content in the microparticle. Similar results by Coccoli et al. (2008) for PCL encapsulation of hydrophilic material were obtained. Moreover, PVA concentration influences the microparticle size. Higher PVA concentration decreases the size of the microparticles from 29 to 25 μm. This effect can be explained since the emulsifier decreases the superficial tension between organic phase drops and the aqueous phase. Similar results with flurbiprofen encapsulated in PHBV were observed (Coimbra et al., 2008)

Therefore, when the emulsifier concentration increase in the aqueous phase, the organic phase drops can easily disperse producing a small size microparticle (run 1, size of 25 μm). Since the average medium size of the microparticles is small, the total superficial area is large, which facilitates the drug molecules escape from the matrix and results in a lower drug encapsulation (0.7% *vs* 2.8%).

The drug initial concentration also affects the drug encapsulation, as is shown in Table I (runs 3 and 4). Maintaining the same PHBV and PVA concentrations (40 mg/mL and 1.0%, respectively) and increasing the RIF concentration (3.9 mg/mL to 10.0 mg/mL), the EE increased from 8.2% to 13.9% and RIF content from 2.5% to 4.8% which indicates that the increase of the RIF initial concentration significantly increases the RIF content in the microparticles. The average size did not change significantly with this variation. The initial drug concentration in the solvent phase was one of the main parameters affecting drug loading. This is an expected result because there are more drug molecules available in the case of higher initial drug concentration which led to higher drug loading in a similar way as that observed in PHB (Kassab et al., 1997). The date in Table I show that this is a promising technique to obtain under optimized

condition, yields over 60% and encapsulation efficiencies over 14%.

RIF release

Fig. 3 shows the *in vitro* drug release of the RIF-loaded PHBV microparticles, which is characterized by an initial burst followed by sustained release. The fast release can be attributed to the large surface area. The small differences between the release rates could be attributed to the microparticle size that is direct related to PHBV concentrations. A large initial PHBV concentrations increase the microparticles size. Thus, a lower rate was observed for large size microparticles (43 μm) which released 93% of the RIF in 24 h and while for small size microparticles (29 μm), the liberation was total in the same period (Table I). The same result was described for drugs encapsulated in solid lipid nanoparticles (Celikkaya et al., 1996). The PVA and RIF concentrations did not significantly influence the drug release. In addition, the faster drug release of PHBV microparticles may be caused by the porosity of the microparticles (Fig. 1). Thus, the drug molecules near to the matrix surface and high porosity facilitate the drug release.

Antimicrobial activity of RIF encapsulated in PHBV microparticles

Table II shows the bacterial growth inhibition areas of the free RIF and PHBV-RIF microparticles in Petri plates inoculated with *Staphylococcus aureus*. The results show that microencapsulation exhibited similar inhibition as the free form acting on *Staphylococcus aureus* at 20 and 24 h incubation.

Cytotoxicity of free RIF and encapsulated in PHBV microparticles

Fig. 4 shows the viability of V79 fibroblasts after treatment with free RIF for 24 h. The cytotoxicity of free RIF for the three endpoints assayed was concentration-dependent. The RIF showed cytotoxicity in the NRU, MTT, and NAC assays with IC_{50} of 325 μM, 425 μM and 175 μM, respectively.

Fig. 5 shows the comparison of RIF free and encapsulated in PHBV microparticles on the concentration at a 400 μM. The observed results show a cytotoxicity reduction with encapsulation of RIF. The microencapsulation reduced the toxicity of the free RIF by 20% in the assay that evaluates the lysosomal integrity (NRU); 10%

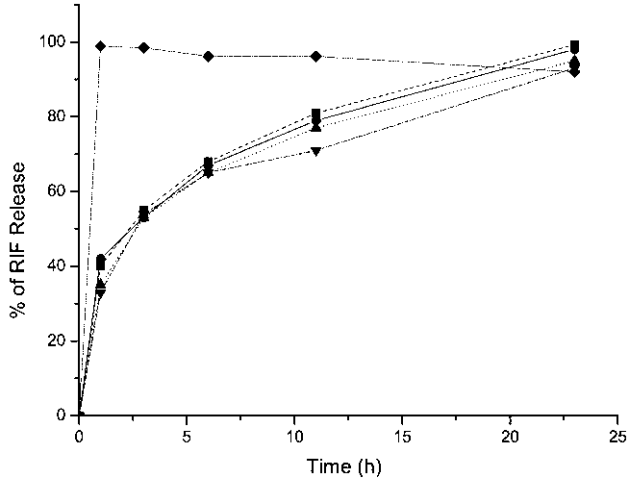

Fig. 3. RIF release profiles of PHBV microparticles and dissolution of free RIF (Table I): Run 1 (- ■ -); Run 2 (- ● -); Run 3 (- ▲ -); Run 4 (- ▼ -); Free RIF (- ◆ -)

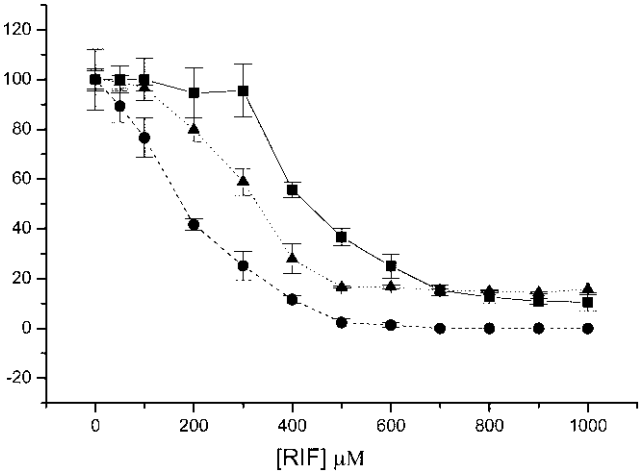

Fig. 4. Cellular viability of V79 cell culture for free RIF in the endpoints of NAC (- ● -), NRU (- ▲ -) and MTT (- ■ -) assays

Table II. Bacterial growth inhibition areas (halo) of *Staphylococcus aureus* by free RIF and microencapsulated in function of time (h)

Sample	[RIF] μg/mL	Halo* (cm) 20 h	% release 20 h	Halo* (cm) 24 h	% release 24 h
RIF free	4.0	4.5±0.2	100±4	4.5±0.3	100±4
RIF microencapsulated run 2[a]	4.0	4.3±0.3	78±3	4.5±0.2	95±3
RIF microencapsulated run 4[b]	4.0	4.1±0.2	74±3	4.1±0.3	90±4

*Values are expressed as mean SD (n = 3).
[a] Run 2 (Table I): PHBV 10 mg/mL, RIF 10 mg/mL, PVA 1.0%.
[b] Run 4 (Table I): PHBV 40 mg/mL, RIF 10 mg/mL, PVA 1.0%.

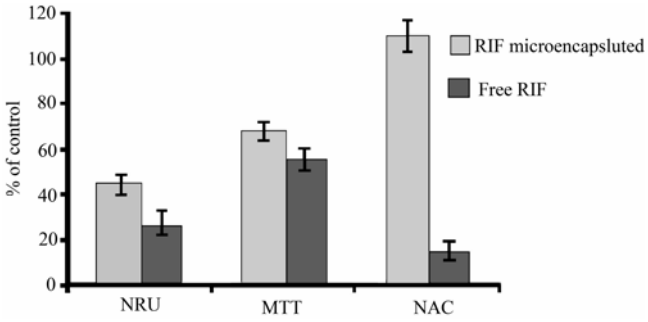

Fig. 5. Comparative graphic of the viability of V79 cells

in the MTT reduction that evaluates the mitochondria integrity and 90% in the nucleic acid content.

CONCLUSION

The PHBV, RIF and PVA concentrations were the main factors in the determination of the size, EE and RIF release from PHBV microparticles.

The EE and yield under optimum conditions (PHBV, PVA, RIF concentration) reached values of 14% and 60%, respectively. Antimicrobial assay together with cytotoxicity studies clearly showed that the encapsulation of RIF in PHBV microparticles maintained the antimicrobial activity and decrease the cytotoxicity of the free RIF. In conclusion, this study demonstrates the potential for the use of PHBV as a biodegradable polymer in antibiotic encapsulation, which can decrease the toxicity of the drug while maintaining its antimicrobial activity.

ACKNOWLEDGMENTS

We acknowledge the financial support from CNPq, FAPESP, Brazilian Network in Nanobiotechnology and Brazilian Network in Nanocosmetics (MCT/CNPq).

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