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Respirable rifampicin-based microspheres containing isoniazid for tuberculosis treatment

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Abstract: The purpose of this investigation was to develop small microspheres for delivering antimycobacterial drugs to infected host macrophages. Rifampicin-based microparticles were prepared. The drug was covalently linked to acrylic moieties to obtain a polymerizable derivative for the preparation of materials useful as drug delivery systems that then were loaded with isoniazid acting in synergy with rifampicin. Their antitubercular activity was determined *in vitro*. Fourier transform infrared spectroscopy confirmed hydrogel structure. Morphological analysis showed microparticles with spherical shape and homogeneous surface. *In vitro* release studies were performed in media simulating physiologic pH (7.4) and endosomal of alveolar macrophages pH (5.2). A similar amount of isoniazid was delivered within the first 6 h at both pHs, while a smaller amount of the drug was delivered at pH 7.4 in the last phase of the study. *In vitro* antitubercular activity showed a behavior comparable to that of rifampicin and isoniazid free. Bioactive swelling matrices, showing a high swelling degree into a medium miming intra alveolar environment, were obtained. They could be applied for their antitubercular activity. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A: 000–000, 2011.

Key Words: rifampicin, isoniazid, microspheres, pH-controlled release, antitubercular activity

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INTRODUCTION

Controlled drug delivery systems, designed to release drugs at predetermined rates and for predefined periods of time, have been used to overcome the shortcomings of conventional drug formulations.[1–3] Hydrogels have emerged as a promising option in this regard.[4–10] They are crosslinked hydrophilic polymeric structures that can imbibe large amounts of water or biological fluids. Lately, stimuli-responsive hydrogels have been studied since they exhibit reversible swelling behavior in response to external stimuli such as pH, temperature, or magnetic and electric field.[11–15] In particular, pH-sensitive hydrogels are widely used because of variations in pH that are known to occur at several body sites such as the pulmonary and gastrointestinal tracts,[16] vagina, and blood vessels. The design of a new biodegradable and biocompatible stimuli-sensitive polymeric system represents an interesting incentive for several researchers.[17–19] It is possible to prepare these materials by using different polymerization techniques. Among these, reverse-phase suspension polymerization method allows to obtain spherical microparticles with a narrow size distribution.[20,21] Spherical shape should be advisable to avoid swelling anisotropic behavior associated with other geometries.[22] The hydrophilicity of beads allowed to incorporate high concentrations of water-soluble drugs into the spheres after synthesis. Employing different model drugs, release profiles from microspheres were studied. In particular, the drug release features depended principally on crosslinking degree of polymers, ratio among functionalized drug and comonomer, and drug–matrix interactions.

Microsphere technology has been used to deliver several different types of drugs, including antigens, steroids, peptides, proteins, and antibiotics, by injection or oral administration.[23–25] The purpose of this investigation was to develop small microspheres for delivery of antimycobacterial drugs to infected host macrophages. For these studies, rifampicin and isoniazid were chosen because they are two of the established first-line drugs used to treat tuberculosis.

Pulmonary *Mycobacterium tuberculosis* infection is characterized by alveolar macrophages containing large numbers of bacilli. Current treatment of pulmonary tuberculosis involves prolonged oral administration of large systemic doses of combined antibiotics, which are associated with unwanted side effects and poor patient compliance.

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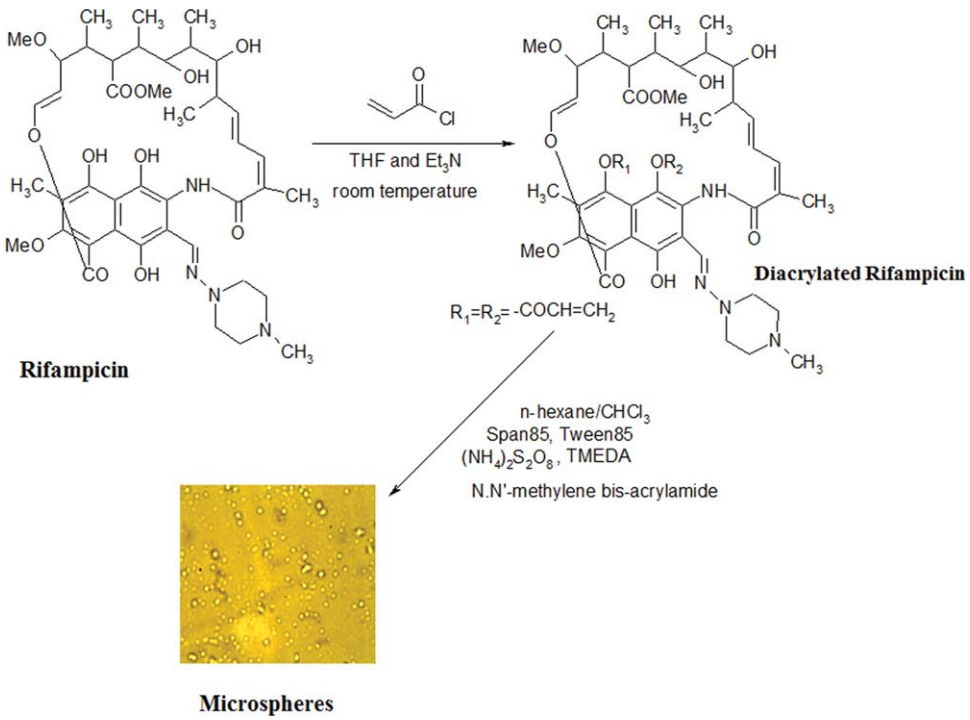

FIGURE 1. Schematic illustration of microspheres rifampicin based preparation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Targeting the drug to alveolar macrophages would be a rational addition to current therapy, potentially enhancing efficacy, and reducing toxicity.[26–28] Recently, several particle technologies have emerged, which have enabled inhaled microspheres to seek to manipulate pulmonary biopharmaceuticals, and to improve therapeutic efficacy for both local and systemic treatments. These microspheres may be designed to sustain drug release, to prolong lung retention, to achieve drug targeting, and/or to enhance drug absorption and thereby, to seek the potentials of reducing dosing frequency and/or drug dose, while maintaining therapeutic efficacy and/or reducing adverse effects. In particular, particles possessing an aerodynamic diameter that fits into the range of 1–3 μm aggregate with difficulty and efficiently deposits in the pulmonary region. Because of their aerodynamic size, these particles will deposit in the periphery of the lung where they will be ingested by alveolar macrophages.[29–32]

To prepare pH-sensitive microspheres, suitable for aerosol drug administration, the present work describes the synthesis of materials by esterification of an antitubercular drug, like rifampicin, with a polymerizable group such as acrylic one for obtaining a bioactive monomer useful for the preparation of swellable hydrophilic microspheres through suspension radical polymerization (Fig. 1).

The beads obtained were characterized by scanning electronic microscopy (SEM), Fourier Transform IR spectrophotometry, particle size distribution analysis, swelling, and drug releasing behavior. The polymeric network showed a pH-dependent behavior. Finally, to obtain information about drug release profile, microparticles were soaked into a solution of isoniazid.

In vitro release studies in simulated pulmonary fluids have shown the influence of the environmental pH on release profiles. The matrix of the beads was also subjected to spectrophotometric investigations concerning its hydrolysis at different pH and the results showed that the matrix releases small amounts of rifampicin and only after about 6 h (data not shown). Moreover, microspheres' antibacterial activity against *Mycobacterium tuberculosis* complex was evaluated.

MATERIALS AND METHOD

Apparatus

The infrared spectra were obtained from KBr pellets using a FT-IR spectrometer Perkin-Elmer 1720, in the range 4000–400 cm^{-1} (number of scans 16). ^1H NMR spectra were processed using a spectrometer Burker VM30; chemical shifts are expressed in δ and referred to the solvent. The structures of the compounds synthesized were confirmed also by GC-MS Hewlett Packard 5972. UV–vis spectra were realized through a UV-530 JASCO spectrophotometer. The light scattering was performed with a Brookhaven 90 plus particle size analyzer. The samples were lyophilized utilizing a "freezing-drying" micro moduly apparatus, Edwards. Scanning electron microscopy (SEM) photographs of the microspheres were obtained with a JEOL JSMT 300 A; the surface of the samples was made conductive by deposition of a gold layer on the samples in a vacuum chamber. Antitubercular activity was evaluated with the Becton Dickinson Detection Instrument (Becton Dickinson, USA).

Materials

All solvents were obtained from Carlo Erba Reagents (Milan, Italy). *N,N′*-dimethylacrylamide (DMAA) and acryloyl chloride, were supplied by Sigma (Sigma Chemical, St. Louis, MO) and distilled before using. Rifampicin, potassium carbonate (K_2CO_3), ammonium persulfate $(NH_4)_2S_2O_8$, sorbitan trioleate (Span 85), polyoxyethylene sorbitan monolaureate (Tween 85), *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) were purchased from Aldrich Chemical and used as received. Middlebrook 7H9 medium was purchased from (Becton Dickinson, USA).

Rifampicin derivatization

The reaction was carried out according to the already known procedure.[33–37] In a three-neck flask fitted with a reflux condenser, dripping funnel, magnetic stirrer, carefully flamed, and maintained in an inert atmosphere, a preset amount of rifampicin (0.5 g, 6.07×10^{-3} mol) was dissolved in 20 mL of tetrahydrofuran (THF) dry. Then we added 0.21 mL (1.48×10^{-3} mol) of triethylamine and the reaction mixture was kept in continuous agitation at a temperature of 25°C. Afterward, acryloyl chloride (0.12 mL,1.48×10^{-3} mol) dissolved in 5 mL of tetrahydrofuran (THF) was added dropwise into the flask. The addition of the acrylate compound caused a change of solution color from dark red to orange. Then the reaction was allowed to reflux for about 12 h and was constantly monitored by TLC on neutral alumina plates (using a mixture chloroform/methanol 9:1, v/v, as the eluent) until the existence of the link between rifampicin and the acrylic group was confirmed. The formation of the product was proved by FT-IR and ^{1}H-NMR.

Microspheres preparation

Rifampicin-based microspheres by radical copolymerization technique were produced. Briefly a mixture of *n*-hexane and chloroform was placed in a round-bottomed cylindrical glass reaction vessel fitted with an anchor-type stirrer and thermostated at 40°C, then treated, after 30 min of N_2 bubbling, with a solution of acrylated rifampicin (100 mg, 1.14×10^{-4} mol), comonomer DMAA (5.7×10^{-5} mol) and ammonium persulfate (800 mg) in water such as radical initiator. The density of the organic phase was adjusted by the addition of $CHCl_3$ or *n*-hexane so that the aqueous phase sank slowly when stirring stopped. Under stirring at 1000 rpm, the mixture was treated with Span85 and Tween85, then filtered, washed with 50 mL portions of 2-propanol, ethanol, acetone, and diethyl ether and dried overnight under vacuum at 40°C until constant weight.[38] Before application, the microspheres were lyophilized to remove residual traces of all utilized solvents.

Size distribution analysis

The size of microparticles was determined by dynamic light scattering (DLLS) using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, NY) at 25°C by measuring the autocorrelation function at 90° scattering angle. Cuvettes were filled with 100 mL of sample solution and diluted to 4 mL with filtered (0.22 mm) water. The polydispersity index (PI), which indicates the measure of the distribution of nanoparticle populations,[39] was also determined. Six separate measurements were made to derive the average. Data were fitted by the method of inverse "Laplace transformation" and Contin.[40,41]

Swelling studies

The swelling behavior was investigated to check the hydrophilic affinity of spherical microparticles. Typically, aliquots (50 mg) of dried materials were placed in a tared 5-mL sintered glass filter (Ø 10 mm; porosity G3), weighed, and left to swell by immersing the filter in a beaker containing the swelling media. The pH values were selected to simulate physiologic pH (7.4) and endosomal pH of alveolar macrophages (5.2).[42] Three replicates were used for each pH value. At predetermined times (1, 6, 12, and 24 h), the excess of water was removed by percolation and then the filter was centrifuged at 3500 rpm for 15 min and weighed. The filter tare was determined after centrifugation with only water. The weights recorded at different times were averaged and used to give the equilibrium swelling degree [wt (%)] by the Eq. (1) where W_s and W_d are the weights of swollen and dried microspheres, respectively. Each experiment was carried out in triplicate and the results were in agreement within ±4% standard error.

$$W_t(\%) = (W_s - W_d)/W_s \times 100 \quad (1)$$

Drug incorporation into preformed microspheres

Incorporation of isoniazid into microspheres was performed as follows: 100 mg of preformed empty microspheres were soaked with 8 mL in a concentrated drug solution (2.5 mg mL^{-1}). The amount of drug dissolved was chosen so as to have a loading equal to 20% p/p. After 3 days, under slow stirring at 37°C, the microspheres were filtered and dried at reduced pressure in presence of P_2O_5 to constant weight. After that, the loading efficiency percentage (LE%) was determined by UV–vis spectroscopy analysis of filtered solvent according to Eq. (2):

$$\mathrm{LE}\% = \frac{M_i - M_0}{M_i} \times 100 \quad (2)$$

where M_i is the mass of drug in the solution prior to loading, M_0 is the mass of drug in solution after loading, monitored by measuring absorbance at a wavelength of 202 nm.

In vitro drug release from microparticles

Dried microspheres (10 mg) were dispersed in 6 mL of swelling media (7.4 physiologic pH and 5.2 endosomal of alveolar macrophages pH). The test tubes were maintained at 37°C in a horizontal-shaking bath and shook at a rate of 100 rpm. At predetermined intervals, the samples were centrifuged, 5 mL of supernatant was removed and the medium was replaced with fresh solution to maintain the same total volume throughout the study. The concentration of isoniazid was determined by UV spectrophotometry at fixed

wavelengths ($\lambda = 202$ nm) employing different molar absorption coefficient depending on the release solutions ($\varepsilon = 170$ L mol^{-1} cm^{-1} for solution with pH = 5.2 and $\varepsilon = 6820$ L mol^{-1} cm^{-1} for solution with pH = 7.4). Each *in vitro* release study was performed in triplicate. Drug release was calculated in terms of percentage of drug released.

In vitro antitubercular activity evaluation

The antitubercular activity of isoniazid-loaded microspheres was tested in Mycobacteria growth indicator tube (MGIT).[40] The MGIT tube contains 7 mL of modified Middlebrook 7H9 broth and in the culture tubes is contained a fluorescent sensor embedded in silicone at the bottom which responds to the concentration of oxygen. Initial concentration of dissolved oxygen quenches the emission from the compound, and little fluorescence can be detected. Actively respiring microorganisms consume the oxygen which allows the compound to release fluorescence. In the isoniazid-loaded microspheres the initial concentration of isoniazid was 2 μg mL^{-1}, the initial concentration of rifampicin was 20 μg mL^{-1}: in a MGIT tube was added in required amount to achieve a concentration of 0.1 μg mL^{-1} for isoniazid and a concentration of 1 μg mL^{-1} for rifampicin (according to chemosensitivity assays *in vitro*) and in the same tube was added 0.5 mL of broth culture positive for *Mycobacterium TB complex*; a second MGIT tube was added with not loaded microparticles to achieve a concentration of 1 μg mL^{-1} (according to chemosensitivity assays *in vitro*) and 0.5 mL of broth culture positive for *Mycobacterium TB complex*, and a third MGIT tube (growth control) was added 0.5 mL of broth culture positive for *Mycobacterium TB complex* diluted 1:100 in distilled water before addition to the control tube (according to chemosensitivity assays *in vitro*). All MGIT tubes were supplemented with 0.8 mL of the provided enrichment (BACTEC MGIT 960 SIRE Supplement; Becton Dickinson). The tubes were placed in MGIT rack in a fixed sequence and the rack was incubated in the instrument MGIT 960 Becton Dickinson detection instrument (Becton Dickinson, USA) until the instrument has signaled the end of the test.

Statistical analysis

All data are presented as means ± SD for three separate experiments. Data were analyzed by two way ANOVA test, followed by Bonferroni's post test, using the GraphPAD Prism4 software (GraphPad Software, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Numerous literature data attest that the focus of current research is directed to the study of carrier molecules capable of promoting the site-specific drug release.[44] Rifampicin is a molecule that has many useful free hydroxyl groups to form ester bonds with acryloyl chloride. The rifampicin hydroxyl groups capable of reacting are those linked to benzene rings, because they have no steric encumbrance. After 24 h, the reaction was monitored on TLC plates with aluminum oxide (using as eluent chloroform and methanol 9:1).

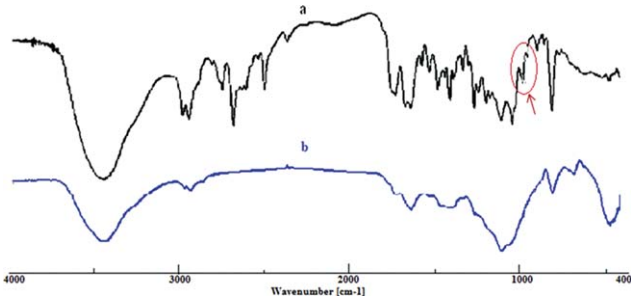

FIGURE 2. IR analysis of (a) diacrylated rifampicin, (b) hydrogel microspheres. Arrow indicates acrylic bands. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The product was dried under vacuum and analyzed by FT-IR. The technique used for the preparation of acrylate rifampicin-based microspheres is the polymerization in suspension. The aqueous solution of monomer (acrylate rifampicin) and comonomer (DMAA), which forms the dispersed phase, was added to an excess of organic solvents (*n*-hexane and chloroform), immiscible with water, that forms the dispersant phase. Under stirring, the dispersed phase forms small droplets that assume a spherical shape to reduce their interfacial free energy. The radical polymerization provides a chain mechanism for growth that begins with the generation of primary radicals following the cleavage of an appropriate initiator (ammonium persulfate). Then, these radicals react with the acrylic functions on the derivatized rifampicin determining its cross-linking. The reaction was started using TMEDA and ammonium persulfate as initiator system. To ensure a greater fragmentation of the phase containing the monomers, the density of the organic phase was adjusted by adding one of the two solvents to obtain an aqueous phase in equilibrium with the organic phase. To prevent the aggregation of spherical particles, the suspension was kept under constant agitation (1000 rpm). The spherical particles are more stable in the organic phase by adding a mixture of surfactants Span85 and Tween85. Optimization of the polymerization method required several attempts. It was observed that hydrophilic/lipophilic balance (HLB) of surfactants is very important. Many tests were carried out to determine the correct ratio for Span85 (HLB = 1.8) and Tween85 (HLB = 11). Finally, we observed that a system, with HLB = 4.8, is able to stabilize the aqueous dispersed phase.

These additives stabilize the system, reducing the surface tension between the particles of monomer and the dispersant phase. The suspension polymerization allowed to obtain stable crosslinks, since covalent bonds formed between the derivatized rifampicin molecules. The obtained materials were characterized by Fourier Transform IR spectrophotometry, swelling behavior, particle size distribution analysis, and morphological analysis. The spectrum of acrylate rifampicin (Fig. 2) clearly shows two bands typical of the acrylic functions at 949 and 976 cm^{-1} that confirm ester-linkages (black curve). These bands disappear in the spectra of drug cross-linked with DMAA (blue curve).

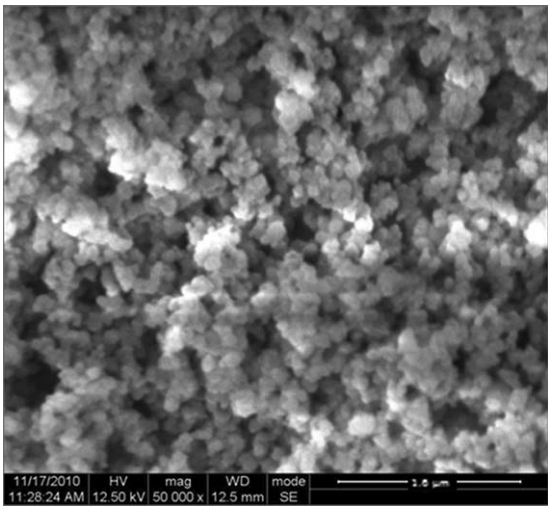

FIGURE 3. SEM photomicrographs of microspheres.

The ^{1}H NMR spectrum showed that the obtained derivative was diacrylated and allowed to locate the position of acrylic groups. ^{1}H NMR ($CDCl_3$), δ (ppm): 8.100 (1H), 7.295 (1H, d), 6.941 (1H, d), 6.921 (1H, dd), 6.899 (1H, dd), 6.453 (1H, dd), 6.401 (1H, dd), 6.399 (1H, dd), 6.294 (1H, dd), 6.084 (1H, dd), 6.082(1H, dd), 5.725 (1H, dd), 4.209 (1H, dd), 3.974 (1H, dd), 3.779 (3H), 3.631 (3H), 3.530 (1H, dd), 3.364 (1H, ddd), 3.364 (1H, ddd), 3.200 (3H), 3.192 (1H, ddd), 3.192 (1H, ddd), 2.826 (1H, dqd), 2.813 (1H, ddd), 2.813 (1H, ddd), 2.636 (1H, ddd), 2.636 (1H, ddd), 2.579 (1H, qdd), 2.521 (1H, dd), 2.402 (3H), 2.214 (1H, dqd), 2.211 (3H), 2.121 (1H, dqd), 1.903 (3H), 1.259 (3H, d), 1.038 (3H, d), 1.027 (3H, d), 1.013 (3H, d).

The observation at the optical microscope of polymerized rifampicin shows the presence of spherical particles (Fig. 1). The shape was confirmed using scanning electron microscopy (SEM) (Fig. 3). In our experiments a mean particle diameter of around 1.6 ± 0.024 μm was obtained.

Investigation of the applicability of these hydrogels in controlled release was done by studying their swelling behavior in media simulating physiologic pH (7.4) and endosomal pH (5.2) of alveolar macrophages at 37°C. Data reported in Table I illustrate the water uptake[45] at each studied pH.

To estimate the ability of the prepared matrices to release drug molecules, the beads were loaded with isoniazid by soaking procedure that allows the drug in contact with the outer surface of the microspheres to establish weak electrostatic interactions with the matrix (predominantly at the surface). However, the chemical nature of the drug and the matrix, as well as the loading time, makes a proportion of active molecules to interact with the core of the microspheres. During the impregnation, the synthesized biopolymer increases in volume but retains its three-dimensional structure without disintegrating because it is insoluble in water. Impregnation requires the interaction of a weighed amount of microspheres in a small volume of a solution at known drug concentration for 72 h. The percentage of adsorbed drug (LE%) was evaluated through a spectrophotometer by measuring absorbance at a wavelength of 202 nm[46,47]; the obtained value showed that the polymer matrices is able to appreciably interact with the drug with loading percentages around 60%. We also carried out the *in vitro* drug release studies at 37°C and both pH 5.2 and pH 7.4 for 24 h. The experimental data showed a constant increase of isoniazid release at pH 5.2 as a consequence of the polymer swelling behavior at this pH, where it increased with time thus enhancing drug mobility and diffusion from the microspheres. As shown in Figure 4, at pH 7.4 lower amounts of isoniazid were released as a consequence of the lower water uptake and swelling in this environment.

TABLE I. Swelling Degree (wt %)of the Hydrogel Microspheres at Different pHs

Time	wt % pH 5.2	wt % pH 7.4
1 h	19	35
2 h	22	38
4 h	170	100
6 h	429	101
12 h	571	156
24 h	584	200

This is due to the different water affinity, of prepared material, at pH 7.4 and pH 5.2. In particular, when the pH is 5.2, the water content was greater than that found at pH 7.4. It is possible to explain this behavior as a consequence of rifampicin pK_a (1.7 and 7.9). Based on these values, at pH 7.4, it is expected to witness a greater absorption of water. However, the results obtained, reported in Table I, indicate the opposite. This is probably due to a partial hydrolysis of the polymer matrix as a result of the formation of phenate ions. This trend also justifies the minor release of isoniazid at pH 7.4 over time. Even at acid pH (1–3) the limited release is due to the formation of inner salts that inhibit the release process.

The antitubercular activity of isoniazid loaded microspheres was determined *in vitro* against *Mycobacterium tuberculosis* complex in Middlebrook 7H9 medium by the instrument MGIT 960 Becton Dickinson Detection Instrument (Becton Dickinson, USA).[43] Analysis of fluorescence in the microparticles-containing tubes compared to the

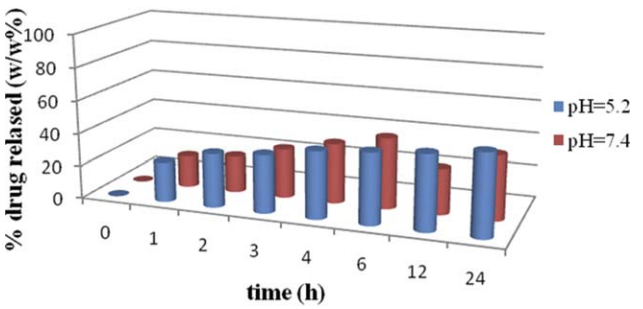

FIGURE 4. Percentage of released isoniazid (%) from the hydrogel microspheres at pH 5.2 and 7.4. Results indicate mean of three independent experiments done in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

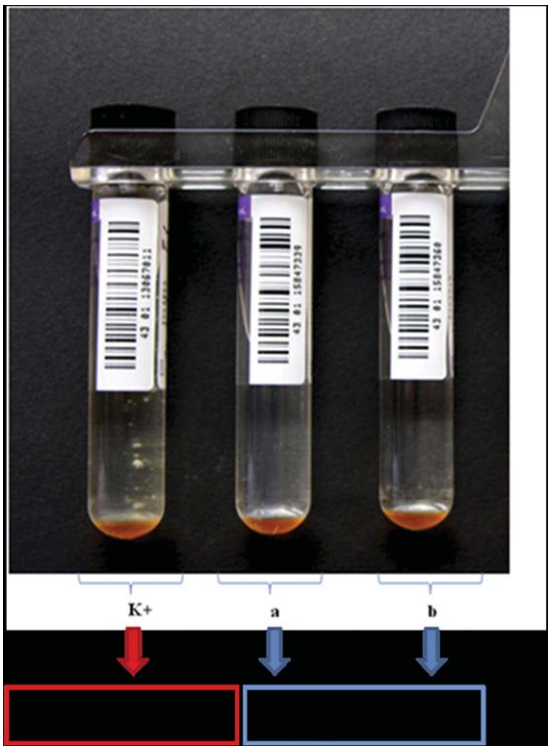

FIGURE 5. *In vitro* inhibition growth of *Mycobacterium tuberculosis* complex in the presence of (a) not loaded microspheres and (b) isoniazid loaded microspheres. The first tube represents the growth control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fluorescence of the growth control tube is used by the instrument to determine susceptibility results.

As shown in Figure 5(b) the isoniazid loaded particles showed antimicrobial activity. The MIC of isoniazid was $\leq$0.1 μg mL^{-1} and for rifampicin $\leq$1 μg mL^{-1}. Not loaded microparticles showed also an activity due to the presence of rifampicin in their structure (MIC $\leq$ 1 μg mL^{-1}) [Fig. 5(a)]. In particular, Figure 5(a) (not loaded microparticles) shows a slight growth (not considered significant for the instrument, indeed *Mycobacterium tuberculosis* complex turned out sensitivity to rifampicin). On the contrary in the Figure 5(b) (isoniazid loaded microspheres) there was no growth, this highlights the drugs synergic action.

CONCLUSIONS

Rifampicin was successfully derivatized by reaction with acryloyl chloride to obtain an active substance which contains chemical groups able to undergo radical polymerization. The beads obtained by radical copolymerization with DMAA, showed spherical shape. The elevated water affinity and the high degree of swelling at pH 5.2, suggests that these materials can be used such as inhalable drug carriers for tuberculosis treatment. To test preformed microspheres as respirable drug carriers, isoniazid was chosen and drug entrapment percentual was determined. The drug release profiles, in media which simulate physiologic as well as endosomal pH of the alveolar macrophages, depend on the hydrogel swelling degree. Concerning to microspheres antibacterial activity data showed a positive behavior of polymers in inhibition of bacteria proliferation. The results suggested that the microparticles posses an excellent antitubercular activity comparable to that of both free rifampicin and isoniazid *in vitro*.[48,49]

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