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RESEARCH ARTICLE

Antimycobacterial activity of 2-phenoxy-1-phenylethanone, a synthetic analogue of neolignan, entrapped in polymeric microparticles

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Abstract

Conventional treatment of tuberculosis (TB) demands a long course therapy (6 months), known to originate multiple drug resistant strains (MDR-TB), which emphasizes the urgent need for new antituberculous drugs. The purpose of this study was to investigate a novel treatment for TB meant to improve patient compliance by reducing drug dosage frequency. Polymeric microparticles containing the synthetic analogue of neolignan, 1-phenyl-2-phenoxiethanone (LS-2), were obtained by a method of emulsification and solvent evaporation and chemically characterized. Only representative LS-2-loaded microparticles were considered for further studies involving experimental murine TB induced by *Mycobacterium tuberculosis* H37Rv ATCC 27294. The LS-2-loaded microparticles were spherical in shape, had a smooth wall and showed an encapsulation efficiency of 93% in addition to displaying sustained release. Chemotherapeutic potential of LS-2 entrapped in microparticles was comparable to control groups. These findings are encouraging and indicate that LS-2-loaded microparticles are a potential alternative to conventional chemotherapy of TB.

Keywords: Synthetic analogue of neolignan, 1-phenyl-2-phenoxiethanone, PLGA microparticles, tuberculosis, antitubercular agent

Introduction

Tuberculosis (TB) remains a major public health problem, causing around 3 million deaths/year[1]. The lack of a standard active regimen against TB, due in part to the long treatment duration (6 months), and the resulting drug toxicity together underline the need for new antituberculous drugs[2]. Although the first-line TB drug regimen still used is more than 40 years old[3] a number of promising compounds have recently been identified[4–6]. The current global situation is characterized by emerging multidrug resistant (MDR) TB and the more recent emergence of extensively drug resistant (XDR)[7]. Two major factors are responsible for the selection of drug resistance strains: (i) inappropriate dosage or duration prescribed and (ii) poor compliance[8].

An effective approach to the treatment of TB is important for reducing its incidence. For instance, a potentially efficient therapeutic strategy for a better management of TB and improved patient compliance could be a reduction in dosing frequency[9]. In effect, microencapsulation has been recently used to accomplish sustained release of drugs and to target drug delivery to the site of action[10].

Polymeric microparticle formulations containing poly (DL-lactide-co-glycolide) (PLGA) are known to be

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biocompatible and biodegradable and have been shown to be an effective drug delivery system capable of sustained release from days to several weeks, depending on the kind of PLGA used[11]. In mice, parenteral administration of PLGA microparticles has exhibited a sustained drug release during 6–7 weeks[12].

PLGA microparticles have been considered as an interesting delivery system to treat TB[13,10,14,15]. Treatment of *Mycobacterium tuberculosis* infected host macrophages with rifampicin (RIF)-loaded microspheres resulted in a significant decrease in the number of viable bacteria, 7 days following initial infection[13]. Thus, a polymeric microparticle system carrying a novel drug might contribute to improve patient compliance and slow down the emergence of drug resistant strains.

Compound 2-phenoxy-1-phenylethanone (LS-2) is a synthetic neolignan analogue that showed promising *in vitro* and *in vivo* antimycobacterial activity on *M. tuberculosis* H37RV[16]. In the present study, PLGA microparticles containing the compound were tested for sustained pharmacological activity in a murine model of TB and also to avoid repeated administrations. The PLGA microparticles were produced by the solvent evaporation technique to be administered parenterally and chemically characterized. Cytotoxicity against experimental murine TB was also assessed.

Methods

Preparation of drug-loaded microparticle

Poly (DL-lactide-co-glycolide) (50:50) resomer RG 505 (MW 70800) was purchased from Boehringer Ingelheim (Germany). Polyvinyl alcohol (PVA), Mowiol 40–88, was obtained from Aldrich Chemical Co. (USA). The analogue of neolignan 2-phenoxy-1-phenylethanone(LS-2) was synthesized by Prof. Dr. Lauro E. S. Barata[17]. Drug-loaded microparticles were prepared following the classical emulsion and solvent evaporation method[18] with slight modifications. Briefly, 60 mL of dichloromethane (DCM) containing the drug and the polymer (polymer:drug, 5:1, w/w) were emulsified into 300 mL of 2% aqueous PVA, v/v at 25°C, by continuous stirring at 600 rpm in an homogenizer (Ika Labortechnik, Germany), until complete DCM evaporation. Loaded microparticles were recovered by centrifugation (15,000×*g*, Himac CR21—Hitachi), washed three times with distilled water, lyophilized during 24 h and stored at 4°C.

Determination of drug content of microparticles

The drug content of each formulation lot was determined by reverse-high performance liquid chromatography using an Shimadzu Instrument (Japan) equipped with a Lichrosphere 100 RP-18 column (125 × 4 mm, 5 µm, Merck, Germany), a 20 µL injector, at a flow rate of 1.0 mL/min and absorbance measured at a wavelength of 240 nm. The mobile phase consisted of Milli-Q water and acetonitrile (2:3, v/v). A series of (LS-2) solutions of known concentrations (ranging from 0.5 to 50.0 µg/mL) was prepared in the solvent mixture used in the mobile phase to generate an analytical curve. To determine drug content in microparticles, a known amount (15.15 mg) was added to 10 mL of a mixture of Milli-Q water and acetonitrile (2:3 v/v), and stirred for 10 min in a vessel. The sample was then filtered in a 0.22 µm filter of regenerated cellulose (Corning®) and analyzed by HPLC.

In order to calculate the encapsulation efficiency (EE) of LS-2 entrapped in PLGA microparticles, the following equation was used:

$$\%EE = \frac{\text{real loaded drug}}{\text{theoretical loaded drug}} \times 100 \quad (1)$$

Size distribution

A small amount of lyophilized microparticles was suspended in Milli-Q water and the size distribution was determined by using a laser diffraction particle size analyzer (SALD 2101, Shimadzu Instruments, Japan). The average diameter was calculated as a mean value of the volume distribution.

Microparticle morphology

Microparticles morphology was determined by scanning electron microscopy. In brief, microparticles placed on a sample stub previously coated with adhesive were coated with gold under vacuum and then examined in a Leica Stereoscan 440 microscope.

In vitro release studies

Drug release from the LS-2-loaded microparticles was carried out in a USP Type II tablet dissolution test apparatus (Hanson SR8-Plus Dissolution Test Station, Hanson Research, USA) at a stirring speed of 100 rpm. The release medium was a mixture of phosphate-buffered saline (PBS) solution at pH 7.4 and absolute ethanol (7:3 v/v) to ensure sinking conditions. Loaded microparticles (6.4 mg) suspended in 500 µL of the same mixture of solvents was placed in dialysis bags (Fisherbrand MW 12,000–14,000, Fischer Scientific, USA) placed in the cubes containing 100 mL of the release medium at 37°C. At selected time intervals, an aliquot of 1 mL was taken from the cubes and 1 mL of fresh medium replaced. The aliquots were analyzed by HPLC as described and the results referred to an analytical curve obtained prior to the start of dissolution tests. Concentrations of the LS-2 ranging from 0.5 to 50.0 µg/mL dissolved in a mixture of PBS solution at pH 7.4 and absolute ethanol (7:3 v/v) were used to construct the reference curve. Each experiment was conducted in triplicate.

Animals

Male specific pathogen-free (SPF) BALB/c mice (4 weeks of age, weighing about 22 g) were purchased from the University of São Paulo and kept in a BSL 3 facility during the experiment. All animal studies were performed according to the Institutional Animal Care and Ethics Rules of the University of São Paulo, Brazil (Protocol #: 06.1.556.53.7).

Experimental infection and chemotherapy studies

Animals were infected by intratracheal route with an inoculum of 5×10^6 viable Colony forming units (CFU) of *M. tuberculosis* H37Rv strain (n.: 27294; ATCC, Rockville, MD, USA), diluted in PBS. Thirty days after infection, mice were treated with the LS-2-entrapped microparticles dispersed in a volume of 100 µL of PBS (at 12.5 or 25 mg/kg) once a week, during 30 days by intramuscular (IM) and subcutaneous (SC) routes. As controls, mice were treated daily with free isoniazid at 25 mg/kg diluted in PBS (Sigma, USA), a first line antituberculous drug, and also with PBS, during the same period and by the same routes. Table 1 summarizes the rationale of the treatment of infected mice. After 30 days of chemotherapy, the animals were killed and the lungs harvested, homogenized, serially diluted and plated on supplemented 7H11 agar media (Difco, USA). CFU were enumerated 21 days post incubation at 37°C with 5% CO_2, and the results expressed as log CFU/mg lung.

Histopathology

For histological analyses, samples of lungs, liver and kidneys of each animal were fixed in 10% buffered formalin and 5 µm sections of each organ stained with hematoxylin-eosin (HE).

Results

Characteristics of the loaded microparticles formulations

Although several formulations of LS-2 entrapped in microparticles were prepared and characterized in this study, only representative ones were considered for further studies involving treatment of infected mice. Various parameters were considered in initial tests of microsphere preparations, including encapsulation efficiency, size, morphology, and *in vitro* release characteristics, which could be improved by adjusting ratios and components of the formulations. The encapsulation efficiency of LS-2 entrapped in PLGA microparticles was 93%, and the particle size analysis exhibited an average size of 5.0 µm and distribution demonstrated a Gaussian curve. Loaded microparticles were spherical in shape and had a smooth wall containing few pores (Figure 1). Figure 2 shows relative amounts of drug released per hour from the LS-2-loaded microparticles into a mixture of PBS solution at pH 7.4 and absolute ethanol (7:3 v/v), at 37°C. Thirty percent of the drug was released in the first 10 h followed by a plateau of sustained release during the experiment.

Table 1. Experimental protocol for treatment of *M. tubeculosis*-infected mice with LS-2-loaded microparticles and controls with PBS or Isoniazid.

Group	Number of animals	Treatment	Route of administration
A	3	PBS	SC
B	3	PBS	IM
C	4	Free INH (25 mg/kg)	SC
D	3	Free INH (25 mg/kg)	IM
E	3	LS-2-loaded microparticles (12.5 mg/kg)	SC
F	2	LS-2-loaded microparticles (12.5 mg/kg)	IM
G	3	LS-2-loaded microparticles (25 mg/kg)	SC
H	3	LS-2-loaded microparticles (25 mg/kg)	IM

PBS, phosphate buffered saline; INH, isoniazid; LS-2, 2-phenoxy-1-phenylethanone; SC, subcutaneous; IM, intra-muscular.

Chemotherapeutic efficacy of the LS-2-loaded microparticles

Figure 3 shows log CFU/mg of mice lungs after 30 days treatment with LS-2-loaded microparticles (12.5 and 25 mg/kg by IM or SC routes) compared to the control groups treated with PBS or free isoniazid by both routes. The chemotherapeutic potential of LS-2 entrapped in

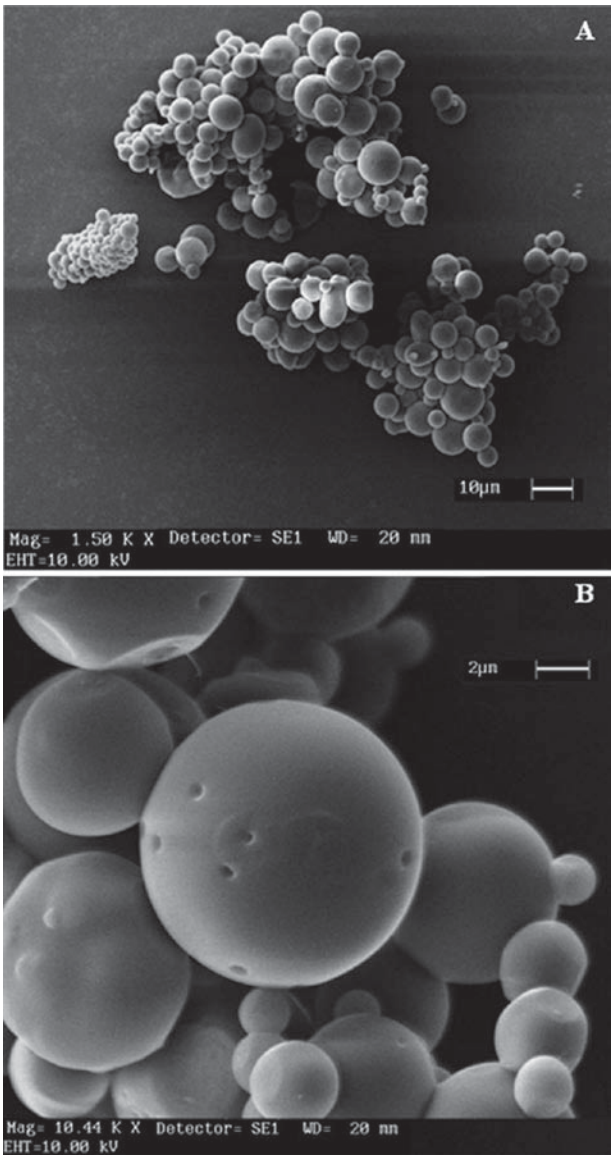

Figure 1. Scanning electron micrographs of LS-2-loaded microparticles in formulations. (A) Taken at a magnification of 1.50 Kx, (B) and at a magnification of 10.44 Kx.

microparticles at 25 mg/kg administered by SC or IM routes was comparable to that of INH, but higher than the controls treated with PBS. However, differences among groups were not statistically significant.

Figure 4 shows 5 µm histological sections of lung tissue stained with HE from groups treated with PBS (A) or with LS-2-loaded microparticles at 25 mg/kg (B).

Discussion

It is assumed that LS-2 entrapped in microparticles could be beneficial against the development of drug resistant organisms, like in TB, by reducing poor patient compliance through shorter therapies. In this therapeutic scheme, drug carrier systems, like LS-2- microparticles, would be capable of sustained release over prolonged time periods, but shorter of classical ones without the need for repeated administration. Systemic side effects would also be significantly alleviated by decreasing the total dose and frequency of drug administration. These attributes make microparticles a promising and ideal alternative for the treatment of TB.

In this study, a polymer-based delivery system carrying a potential new drug for the treatment of TB was obtained by the emulsion and solvent evaporation technique. Encapsulation efficiency of LS-2 in PLGA microparticles was 93% and the particle size analysis exhibited an average size of 5.0 µm, consistent with the findings reported in the literature. The *in vitro* release of LS-2 from microparticles indicated a sustained release for the period evaluated. Moreover, PLGA polymers biodegrade by undergoing random hydrolytic mechanism of their ester linkages[11]. According to Lee et al.[19], surface hydrolysis may occur at different rates than the interior because of factors controlling water penetration and therefore the drug release kinetics from drug/biodegradable matrices are found to be further complicated because of both polymer erosion and drug diffusion. The chemical reaction and mass transfer process controlling drug release from polymeric microparticles depend on the specific device characteristics[20]. A theoretical model of erosion proposed by Batycky et al.[21] indicates that as the microparticles degrades, drug is released by desorption and diffusion. Desorption is due to drug initially contained on the device surface and the mesopores connected to the external

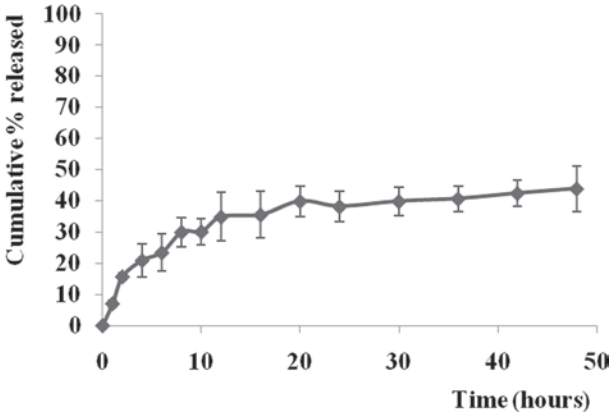

Figure 2. *In vitro* drug release per hour from LS-2-loaded microparticles into phosphate buffered saline (PBS), pH 7.4, mixed with absolute ethanol (7:3 v/v) at 37°C. Error bars indicate ± SD ($n = 3$).

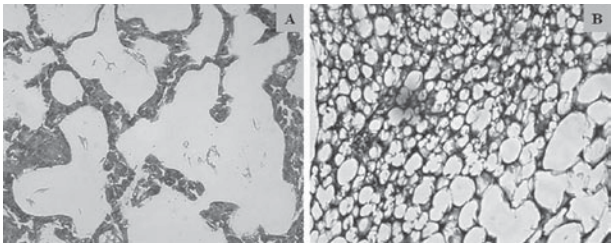

Figure 4. Lungs histopathology of mice experimentally infected with *M. tuberculosis* H37Rv and treated with PBS (A) and with LS-2-loaded microparticles at 25 mg/kg (B). Samples were stained with hematoxilin-eosin.

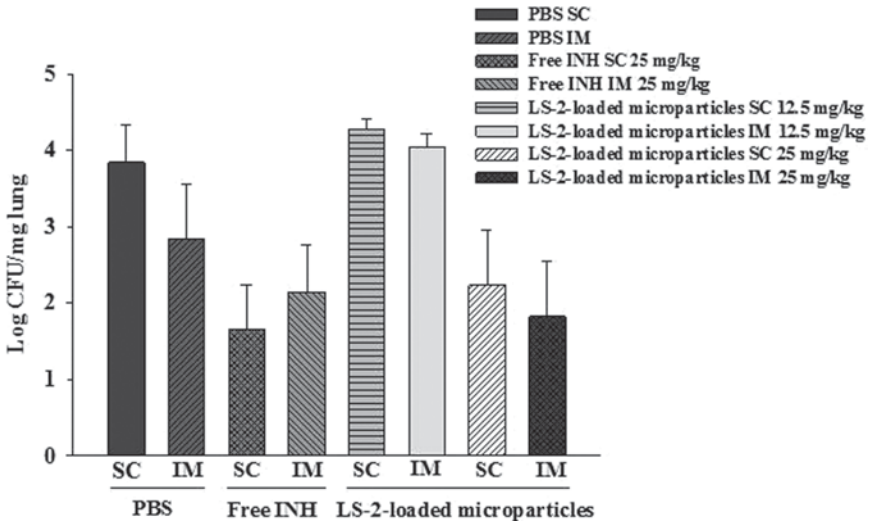

Figure 3. Lung colony forming units (CFU) from experimentally infected mice with *M. tuberculosis* H37Rv after treatment with PBS, Isoniazid (INH) at 25 mg/kg and LS-2-loaded microparticles at 12.5 and 25 mg/kg. All values are means ± standard error of experiments with three or four animals.

surface of the microparticles. Drug diffusion is delayed by an induction time sufficient to allow for micropores to coalesce and permit the passage of the drug.

It was macroscopically observed that particles remained at the site of injection forming a depot from where the entrapped contents of the polymeric system were released. These findings are in contrast with the results reported by Barrow, showing that microspheres with a size distribution of less than 10 μm were phagocytosed by macrophages[13], probably because that study was carried out *in vitro*.

Microparticles carrying LS-2 and administered at 25 mg/kg effectively reduced CFU produced by homogenates of mice lungs when compared with groups receiving daily free isoniazid and were not toxic as observed by histopathological studies.

Lung tissues from all treated groups (including controls) had characteristic lesions in the perivascular sites and peripheral parenchyma showing large sheets of epithelioid macrophages and foamy cytoplasm. Evident granulomas with caseation and lymphoid aggregates were also observed. There were no morphological significant differences in the lung tissues of the group treated with LS-2-loaded microparticles compared with the PBS treated ones. No histopathological differences were observed in liver and kidney tissues.

Conclusions

It is concluded that LS-2 entrapped in polymeric microparticles reduces the CFU of *M. tuberculosis* H37RV in animal lungs. These preliminary results are encouraging and indicate that LS-2 loaded-microparticles are a potential alternative to conventional chemotherapy of TB.

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Declaration of interest

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References

1. Silva CL, Bonato VL, Coelho-Castelo AA, De Souza AO, Santos SA, Lima KM et al. (2005). Immunotherapy with plasmid DNA encoding mycobacterial hsp65 in association with chemotherapy is a more rapid and efficient form of treatment for tuberculosis in mice. Gene Ther, 12:281–287.
2. Ibrahim M, Andries K, Lounis N, Chauffour A, Truffot-Pernot C, Jarlier V et al. (2007). Synergistic activity of R207910 combined with pyrazinamide against murine tuberculosis. Antimicrob Agents Chemother, 51:1011–1015.
3. Rivers EC, Mancera RL. (2008). New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. Drug Discov Today, 13:1090–1098.
4. Haagsma AC, Abdillahi-Ibrahim R, Wagner MJ, Krab K, Vergauwen K, Guillemont J et al. (2009). Selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards the eukaryotic homologue. Antimicrob Agents Chemother, 53:1290–1292.
5 .Nikonenko BV, Protopopova M, Samala R, Einck L, Nacy CA. (2007). Drug therapy of experimental tuberculosis (TB): Improved outcome by combining SQ109, a new diamine antibiotic, with existing TB drugs. Antimicrob Agents Chemother, 51:1563–1565.
6. Thompson AM, Blaser A, Anderson RF, Shinde SS, Franzblau SG, Ma Z et al. (2009). Synthesis, reduction potentials, and antitubercular activity of ring A/B analogues of the bioreductive drug (6S)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5H-imidazo[2,1-b][1,3]oxazine (PA-824). J Med Chem, 52:637–645.
7. Lenaerts AJ, Degroote MA, Orme IM. (2008). Preclinical testing of new drugs for tuberculosis: Current challenges. Trends Microbiol, 16:48–54.
8. Di Perri G, Bonora S. (2004). Which agents should we use for the treatment of multidrug-resistant *Mycobacterium tuberculosis*? J Antimicrob Chemother, 54:593–602.
9 .Sharma A, Pandey R, Sharma S, Khuller GK. (2004). Chemotherapeutic efficacy of poly (DL-lactide-co-glycolide) nanoparticle encapsulated antitubercular drugs at sub-therapeutic dose against experimental tuberculosis. Int J Antimicrob Agents, 24:599–604.
10 .Barrow EL, Barrow WW, Quenelle DC, Westbrook L, Winchester GA, Staas JK. (2007). Efficacy of rifabutin-loaded microspheres for treatment of Mycobacterium avium-infected macrophages and mice. Drug Deliv, 14:119–127.
11. Anderson, JM, and Shive, MS. (1997). Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev, 28: 5–24.
12. Dutt M, Khuller GK. (2001). Chemotherapy of *Mycobacterium tuberculosis* infections in mice with a combination of isoniazid and rifampicin entrapped in Poly (DL-lactide-co-glycolide) microparticles. J Antimicrob Chemother, 47:829–835.
13. Barrow EL, Winchester GA, Staas JK, Quenelle DC, Barrow WW. (1998). Use of microsphere technology for targeted delivery of rifampin to *Mycobacterium tuberculosis*-infected macrophages. Antimicrob Agents Chemother, 42:2682–2689.
14. O'Hara P, Hickey AJ. (2000). Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: Manufacture and characterization. Pharm Res, 17:955–961.
15. Quenelle DC, Staas JK, Winchester GA, Barrow EL, Barrow WW. (1999). Efficacy of microencapsulated rifampin in *Mycobacterium tuberculosis*-infected mice. Antimicrob Agents Chemother, 43:1144–1151.
16. De Souza AO, Alderete JE, Minarini PRR, Da Silva Melo P, Barata LES, Silva CL. (2011). Structure activity relationship, acute toxicity and cytotoxicity of antimycobacterial neolignan analogues. J Pharm Pharmacol, 63:936–942.
17. Barata LE, Santos LS, Ferri PH, Phillipson JD, Paine A, Croft SL. (2000). Anti-leishmanial activity of neolignans from Virola species and synthetic analogues. Phytochemistry, 55:589–595.
18. Bodmeier R, McGinity JW. (1987). The preparation and evaluation of drug-containing poly(dl-lactide) microspheres formed by the solvent evaporation method. Pharm Res, 4:465–471.
19. Lee JW, Gardella JA Jr, Hicks W Jr, Hard R, Bright FV. (2003). Analysis of the initial burst of drug release coupled with polymer surface degradation. Pharm Res, 20:149–152.
20. Yang S, Washington, C. (2006). Drug release from microparticulate systems. In: Benita S, ed. Microencapsulation: Methods and Industrial Applications. New York: CRC Press, pp. 183–211.
21. Batycky RP, Hanes J, Langer R, Edwards DA. (1997). A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. J Pharm Sci, 86:1464–1477.