Gentamicin-loaded microspheres for reducing the intracellular Brucella abortus load in infected monocytes

Sandra Prior1†, Bruno Gander2, Concepción Lecároz1, Juan M. Irache3 and Carlos Gamazo1*

Departments of 1Microbiology and 3Pharmacy and Pharmaceutical Technology, University of Navarra, 31008 Pamplona, Spain; 2Institute of Pharmaceutical Sciences, ETH, 8057 Zürich, Switzerland

Received 20 November 2003; returned 15 January 2004; revised and accepted 8 March 2004

Objectives: The intracellular antibiotic efficiency of gentamicin-loaded microspheres in the context of Brucella-infected murine monocytes was examined in vitro with a view to developing improved therapies for the treatment of brucellosis.

Methods: Biodegradable microspheres made of end-group capped and uncapped poly(lactide-co-glycolide) 50:50 (PLGA 50:50 and PLGA 50:50H) and containing gentamicin sulphate were used to target Brucella abortus-infected J774 monocyte-macrophages. The infected cells were treated with 15 µg of free or microencapsulated gentamicin and the efficacy of the treatments was measured after 24 h.

Results: The particle sizes were below 8 µm and in vitro release of gentamicin from the microspheres followed a continuous (PLGA 50:50H) or a multiphasic (PLGA 50:50) pattern over 50 days. Treatment with gentamicin microencapsulated into the end-group uncapped PLGA 50:50H microspheres, decreased significantly the number of intracellular bacteria (typically by 2 log10) in comparison with untreated infected cells. Addition of 2% poloxamer 188 to the microsphere dispersion medium further reduced the infection (3.5 log10). Opsonization of the particles with non-immune mouse serum had no effect on the antibacterial efficacy of the microspheres. End-group capped PLGA 50:50 type microspheres containing the antibiotic were less effective at reducing intracellular bacteria (∼1 log10 reduction), although addition of poloxamer 188 to the dispersion medium again enhanced their intracellular antibacterial activity. Placebo PLGA 50:50 and PLGA 50:50H microspheres had no bactericidal activity.

Conclusions: The results indicate that PLGA 50:50-microencapsulated gentamicin sulphate may be suitable for efficient drug targeting and delivery to reduce intracellular Brucella infections.

Keywords: biodegradable microspheres, drug delivery systems, Brucella-infected monocytes

Introduction

Brucellosis is an infectious disease caused by Brucella spp. Four species, Brucella abortus, Brucella melitensis, Brucella suis and Brucella canis, have been recognized as human pathogens each associated with a different natural host animal. These small cocobacilli are mainly localized intracellularly within phagocytic cells making treatment difficult, since most antibiotics, although highly active in vitro, do not actively pass through cellular membranes.1 Treatment for brucellosis remains controversial and requires prolonged therapy with at least two agents. The choice of regimen and duration of therapy is based on the presence of focal disease, underlying patient conditions and age group which may contraindicate certain antibiotics. Attempts at monotherapy with trimethoprim/sulfamethoxazole, macrolides or fluoroquinolones have met with disappointing relapse rates despite excellent in vitro activity.2 A prolonged administration of a tetracycline–aminoglycoside combination (i.e. doxycycline 100 mg twice/day for 45 days, and streptomycin 1 g/day for 14 days or gentamicin 5–6 mg/kg per day for 7 days) has lower relapse rates than the more usual doxycycline–rifampicin combination recommended by the World Health Organization in uncomplicated cases.2 An extended doxycycline–aminoglycoside regimen is also the preferred therapeutic option in life-threatening forms of the disease, such as endocarditis.3 However, 3–5% relapse rates, serious side effects (especially in children and pregnant women), and hampered patient compliance, mainly caused

†Present address. Division of Bacteriology, National Institute of Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.
*Corresponding author. Tel: +34-948-425688; Fax: +34-948-425649; E-mail: cgamazo@unav.es

981
by the long-term treatment and the inconvenience of parenteral administration of aminoglycosides, still represent major hurdles contributing to low therapeutic efficacy.\textsuperscript{2}

Gentamicin sulphate is an aminoglycoside with a wide spectrum of antibacterial activity though important side effects mostly related to nephrotoxicity and ototoxicity restrict its use.\textsuperscript{3} It is a highly soluble drug but does not cross cell membranes efficiently, which is an important drawback for the therapy of intracellular infections such as brucellosis, due to the low antibiotic levels achievable inside infected cells. Several reports indicate that gentamicin is more active in vitro against clinical isolates of \textit{Brucella} than streptomycin.\textsuperscript{4} In combined doxycycline–aminoglycoside regimens, gentamicin appears to be more cost-effective and less toxic given the duration of the administration (14–21 days for streptomycin compared with 7 days for gentamicin), with no increases in clinical relapse or treatment failure.\textsuperscript{2} These properties make gentamicin an attractive candidate for the treatment of brucellosis provided that the antibiotic can be delivered intracellularly. Therefore, an optimum strategy to treat brucellosis should target a highly active drug to the intracellular compartment and prolong the release of that antibiotic, thereby reducing the number of doses to be administered and minimizing drug side effects.

Liposomes containing gentamicin are quite efficient for targeting \textit{Brucella}-infected monocyte-macrophages.\textsuperscript{5} Liposomes have a membrane-like structure that favours a good cell interaction and their versatility in terms of structure and composition grant their main advantages. However, liposomes suffer from low encapsulation efficiency, stability problems, both during storage and upon injection, their therapeutic efficacy is not always optimal and cannot provide a controlled release of the encapsulated drug.\textsuperscript{6} Small biodegradable microspheres are attractive alternatives to liposomes for targeting drug in the monocyte-macrophagic system. Upon phagocytosis of antibiotic-loaded microspheres, intracellularly located and highly protected bacteria, such as \textit{Mycobacterium tuberculosis}, \textit{Salmonella typhimurium}, \textit{Listeria monocytogenes} and \textit{Brucella} spp., may become accessible to antibiotic treatment. Biodegradable microspheres made of poly(lactide) (PLA) and its copolymers with glycolide (PLGA) can release encapsulated drugs in a sustained manner, depending on the physicochemical properties of the polymer and drug, and the microencapsulation procedure.\textsuperscript{7} Microsphere technology has been widely used for a wide variety of therapeutic compounds including antibacterial agents.\textsuperscript{8–11} Thus, PLA/PLGA microspheres should be a potentially suitable and stable delivery system for antibiotic treatment of brucellosis that could provide controlled release of the encapsulated drug and minimize the need for multiple injections.

In this study, we examined the intracellular antibiotic efficiency of gentamicin-loaded microspheres in the context of \textit{Brucella}-infected murine monocytes. Two microsphere types, made of end-group capped PLGA 50:50 and uncapped PLGA 50:50H, were used because they are phagocytosed efficiently by monocytes \textit{in vitro};\textsuperscript{12} the end-group uncapped PLGA 50:50H microspheres also promote cell activation, as measured by oxidative burst.\textsuperscript{12,13}

\section*{Materials and methods}

\subsection*{Preparation of microspheres loaded with gentamicin}

Gentamicin was microencapsulated by spray-drying into poly(D,L-lactide-co-glycolide) carrying either capped, i.e. esterified end-groups, or uncapped, i.e. free hydroxyl and carboxyl end-groups,\textsuperscript{14} hereafter abbreviated as PLGA 50:50 and PLGA 50:50H, respectively. Briefly, 100 mg of gentamicin was dissolved in 1 mL of PBS (67 mM, pH 7.4 or 6.0), which was then dispersed in 20 g of a 5% (w/w) polymer solution in ethyl formate by ultrasonication under cooling on ice. The water-in-oil emulsion formed was spray-dried (Mini Spray Drier B-190; Büchi, Flawil, Switzerland) and the resulting microspheres were collected on a cellulose acetate filter of 0.8 \textmu m pore size. Microspheres were washed with 0.1% (w/w) polyoxamer 188 solution, rinsed with distilled water and dried under vacuum (∼10 mbar). Residual water was eliminated by dispersing the particles in n-hexane, followed by additional vacuum drying for 24 h. The final product was stored under dry conditions at 4°C. Placebo microspheres were produced accordingly, but without the gentamicin solution.

\subsection*{Determination of microsphere size and morphology}

Microsphere size distribution was determined by laser light scattering (Mastersizer X, Malvern Instruments, Malvern, UK). The morphology of the microspheres was examined in a Zeiss DSM 940A scanning electron microscope.

\subsection*{Gentamicin content in the microspheres}

Gentamicin content in the microspheres was determined by dissolving 40 mg of particles in 3 mL of dichloromethane and collecting the undissolved gentamicin on regenerated cellulose filters of 0.2 \textmu m pore size. The dried filters were transferred into test tubes and the antibiotic was eluted with 2 mL of water and assayed photometrically (320–350 nm) after derivation with o-phthalaldehyde.\textsuperscript{14} Gentamicin extraction from the microspheres was carried out in triplicate and the eluted gentamicin was determined from the replicates.

\subsection*{In vitro release of gentamicin from microspheres}

Gentamicin release was determined by suspending 10–30 mg of microspheres, weighed accurately, in 4 mL of PBS (67 mM, pH 7.4) containing 0.05% polysorbate 20 and 0.02% sodium azide, in borosilicate vials (Chromacol, Welwyn Garden City, UK). The vials were briefly sonicated to facilitate wetting of the particles and placed horizontally on a shaking platform at 37°C. At regular intervals, the vials were centrifuged at 2000g for 10 min to obtain a particle-free supernatant that was assayed fluorometrically with a Cytofluor 2300/2350 (Millipore)\textsuperscript{5} after derivation of the gentamicin with o-phthalaldehyde. At every sampling point, the release medium collected was replaced with fresh phosphate buffer. Burst release was described as the amount of drug released after 24 h. Gentamicin release from the microspheres was determined in triplicate for each microsphere preparation.

\subsection*{J774 murine monocyte-macrophage cell line}

The J774.2 murine monocyte-macrophage cell line was obtained from the European Collection of Cell Cultures (ECACC No. 85011428). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal calf serum and preserved with 0.1% antibiotic-antimycotic solution (complete medium) at 37°C, 5% CO\textsubscript{2} and controlled humidity.

\subsection*{Brucella strain and preparation of bacteria for infection}

\textit{B. melitensis} is the more frequent cause of \textit{Brucella} human infections. However, as it is a class three pathogen, \textit{B. abortus} was chosen for these studies. \textit{B. abortus} 2308 was isolated from spleens of infected mice by homogenization of the organ in saline solution (Stomacher 80 Lab Blender; Seward Medical, London, UK) and seeding onto trypticase soy agar (TSA) plates (bioMérieux, France). Further, the bacterial colonies were suspended on skimmed milk and maintained in cryovials at −85°C.
Microspheres to treat Brucella-infected monocytes

For monocyte infection, the brucellae were thawed, seeded onto TSA plates and incubated for 2–3 days at 37°C. Two or three isolated colonies were transferred into 5 mL of trypticase soy broth (TSB) and incubated for 20–24 h at 37°C under shaking, so that the bacteria reached exponential growth phase. The bacterial suspension was first diluted in TSB to an optical density of 0.12 (590 nm) and further diluted in saline solution to a concentration of ∼5×10^6 CFU/mL to infect the cultured monocytes.

Infection of monocytes with Brucella abortus 2308

J774 murine monocytes cultured in complete medium were washed, collected by centrifugation and cell viability was determined by Trypan Blue exclusion. Cell numbers were adjusted to 2 × 10^5 cells/mL and 500 µL of the cell suspension was added to each well of a 24-well culture plate (Nunc, Naperville, IL, USA). The cells were incubated in DMEM supplemented with L-glutamine and 10% (v/v) fetal calf serum for 2–3 h to allow cell adherence. Afterwards, the medium was withdrawn to remove non-adherent cells and replaced. For bacterium-specific opsonization, the B. abortus suspension, previously adjusted to an adequate bacterial concentration as described above, was shaken with a sub-agglutinating concentration of antiserum from B. abortus-infected mice for 30 min at 37°C. Then, 100 µL of opsonized B. abortus was added to the monocytes at a ratio of 5 bacteria per cell and further incubated at 37°C for 1–48 h. After incubation, the culture medium was discarded, the infected cells were carefully washed five times to remove extracellular bacteria and 500 µL of DMEM was added to each well. The number of remaining extracellular bacteria in each well was determined by diluting and seeding the added medium onto TSA plates. The total number of CFU per well was determined after lysing the monocytes with cold 0.2% Triton X-100 for 20 min at 37°C, thorough homogenization and plating of serial dilutions of the lysates onto TSA plates. Finally, an inoculum of 10^5 CFU per well was added into B. abortus-infected J774 monocytes and cell viability was determined by Trypan Blue exclusion.

Treatment of Brucella-infected monocytes with gentamicin-loaded microspheres

The infected J774 monocytes were treated with 15 µg of free gentamicin sulphate in solution, with placebo microspheres, or with gentamicin-loaded microspheres containing 15 µg of the antibiotic, and the effectiveness of the different treatments in reducing the number of intracellular bacteria was determined. The influences of prior opsonization of the particles in fresh mouse serum and the presence of 2% (v/v) of the non-ionic surfactant poloxamer 188 in the microsphere dispersion medium were also determined. For opsonization, the microspheres were dispersed in DMEM supplemented with L-glutamine and containing 8% (v/v) non-immune fresh mouse serum and incubated at 37°C under shaking for 30 min. In some experiments, the microspheres were simply dispersed in the 8% (v/v) serum solution without incubation.

The J774 monocytes-macrophages were infected by incubating the cells with the opsonized B. abortus for 15 h at a bacterial/cell ratio of 5:1 as described above. Afterwards, non-phagocytosed bacteria were removed by washing carefully five times and the effectiveness of the different treatments was studied by incubating the infected monocytes for 24 h at 37°C and 5% CO₂ with 1 mL of DMEM/L-glutamine containing either free gentamicin in solution, gentamicin encapsulated in microspheres or placebo microspheres. DMEM/L-glutamine without fetal calf serum was used to avoid potential effects of the serum proteins on the particle uptake and to moderate the proliferation of monocytes and bacteria. Further, cells were washed twice with DMEM/L-glutamine and extracellular CFU and total CFU after cell lysis with Triton X-100 were determined by plating serial dilutions onto TSA plates as described previously. Intracellular brucellae were calculated by subtracting the number of extracellular bacteria from the total CFU counts in three wells. Results were expressed as log₁₀ of intracellular Brucella surviving the treatments and the efficiency of the treatments was tested in one to six independent assays.

In vitro susceptibility of B. abortus to gentamicin and poloxamer

Gentamicin sulphate and poloxamer 188 as single agents or in combination were tested for their ability to inhibit the bacterial growth of B. abortus in broth culture. Gentamicin sulphate was added to the bacterial suspension to obtain final concentrations of 0.007–0.960 µg/mL, alone or in combination with 0.5, 1 and 2% (w/v) poloxamer 188. Bacterial growth in TSB was measured turbidimetrically with an automated Labsystems Bioscreen (Finland) using Bioscreen microplate titrator (100-well honeycomb). Organisms were retrieved from suspensions stored at −85°C in cryoballs (Microbrand, Biolab Diagnostics, Canada), plated on TSA and incubated at 37°C overnight. Several colonies were then subcultured in TSB at 37°C for 36 h to reach mid-log phase. The final inoculum suspension was prepared in TSB and adjusted to an optical density of 0.12 (590 nm) (−10 CFU/mL). The real inoculum dose was estimated by plating appropriate dilutions of the stock suspension onto TSA plates. Finally, an inoculum of 10^5 CFU per well was added into Bioscreen plates containing serial dilutions of the agents (gentamicin and/or poloxamer 188). Appropriate positive and negative growth controls were also included. The optical density of each well was measured automatically at 30 min intervals using wide band irradiation (420–580 nm) and bacterial growth curves were generated.

Statistical analysis

Comparisons were made between groups by a one-way analysis of variance (ANOVA) and post-hoc Tukey’s HSD test. The statistical significance level was defined as P<0.05.

Results

Microsphere size, gentamicin loading and in vitro release

The average diameter of the PLGA 50:50H and PLGA 50:50 microspheres was ∼4 µm, except for the placebo PLGA 50:50 microspheres which had an average diameter of 0.5 µm (Table 1). This size appeared adequate for cellular uptake and was mainly a result of the encapsulation method used. Scanning electron micrographs showed spherically shaped and regular particles with smooth surfaces for both the placebo and gentamicin-loaded PLGA 50:50 microspheres. Gentamicin-loading PLGA 50:50H microspheres were, however, porous and the particles tended to aggregate.

The actual gentamicin loading was 22 and 15 µg/mg microspheres and the in vitro gentamicin burst release amounted to 7.8 and 2.0 µg for the PLGA 50:50 and PLGA 50:50H, respectively (Table 1). Additional gentamicin release from PLGA 50:50H microspheres occurred in a continuous fashion and from PLGA 50:50 microspheres in a multiphase-like pattern (Figure 1).

Kinetics of Brucella uptake and replication in cultured J774 monocytes

Opsonized B. abortus were incubated from 1 to 48 h with J774 cells in complete medium without antibiotic-antimycotic supplement and afterwards, the number of extracellular and intracellular bacteria was determined. Incubation for 1 h was insufficient for significant phagocytosis of opsonized bacteria at a monocyte/Brucella ratio of 1:5. Incubation times between 15 and 48 h allowed an adequate number of
intracellular brucellae to carry out our study (Figure 2). In this time, the bacteria reached the intracellular compartment and multiplied within the monocytes increasing by approx 1.3 log10 after 48 h. Incubation times above 48 h resulted in excessive cell and bacteria proliferation, as reflected by turbidity and diminished cell viability. Therefore, for all antibiotic activity assays, an infection time of 15 h was chosen. Thus, the cells were incubated with the bacteria for 15 h, non-phagocytosed bacteria were washed off and the different treatments were subsequently applied. Under these conditions, the percentage of intra- and extracellular Brucella at the time of the antibiotic treatments was 93–95% and 5–7%, respectively.

Efficacy of gentamicin-loaded microspheres in reducing intracellular Brucella from infected monocytes: effect of polymer type

Monocytes infected with Brucella were treated for 24 h with (i) free gentamicin in solution, (ii) gentamicin encapsulated in PLGA 50:50 or PLGA 50:50H microspheres, and (iii) placebo PLGA 50:50 or PLGA 50:50H microspheres. The amounts of microspheres used per well were 0.7 mg for PLGA 50:50 and 1.0 mg for PLGA 50:50H, which corresponded to a gentamicin dose of 15 µg/well for both
Microspheres to treat *Brucella*-infected monocytes

Microsphere types; corresponding amounts of placebo microspheres were used. A dose of 15 µg of gentamicin was used to ensure the intracellular delivery of the antibiotic at levels well above the MBC (0.06 mg/L, see below). Placebo microspheres had no bactericidal effect on intracellular *Brucella* counts. In contrast, treatment with gentamicin-loaded microspheres reduced intracellular bacteria, compared with untreated controls, by 1.0 and 2.0 log_{10} for PLGA 50:50 and PLGA 50:50H, respectively. Free gentamicin in solution exerted variable intracellular bactericidal activity in different experiments, varying from a 1.1 to 1.8 log_{10} reduction. However, treatment with gentamicin-loaded PLGA 50:50H microspheres was significantly more effective at reducing the intracellular infection than free gentamicin treatment (Figure 3).

**Efficacy of gentamicin-loaded microspheres in reducing intracellular *Brucella* from infected monocytes: effect of particle opsonization and addition of poloxamer 188 to the culture medium**

Previous results in our laboratory showed that opsonization of gentamicin-loaded microspheres increased significantly the capacity of the particles to induce oxidative burst of monocytes, which may potentially increase the bactericidal activity of antibiotic treatments. With that hypothesis, the effect of opsonization on the antibacterial efficacy of the microsphere treatment was examined in this study. Our hypothesis was, however, not supported by our findings. The antibiotic activity of the gentamicin-loaded microspheres did not increase upon particle opsonization (Figure 3). In contrast, addition of 2% (w/v) of poloxamer 188 to the medium to improve the dispersion characteristics of the microspheres, enhanced significantly the intracellular antibiotic activity of the gentamicin-loaded PLGA 50:50H microspheres (Figure 4). In the presence of the surfactant, the total reduction of intracellular bacteria reached 3.5 log_{10} cfu, relative to the untreated monocytes, which represented a 1.2 log_{10} further reduction in intracellular cfu relative to the same treatment without poloxamer 188. A similar improvement, i.e. ~1 log_{10} further reduction, was also observed with the gentamicin loaded end-group capped PLGA 50:50 microspheres when the poloxamer was added to the medium; however, in this case the increase was not statistically significant. Control wells demonstrated the lack of activity of both a poloxamer 188 solution and a suspension of placebo microspheres in poloxamer on the viability of the intracellular *Brucella*.

**In vitro susceptibility of *Brucella abortus* to gentamicin and poloxamer**

The effect of combining gentamicin sulphate and poloxamer 188 against *B. abortus* was studied in broth culture in vitro. When sub-MIC concentrations of gentamicin sulphate and non-inhibitory concentrations of poloxamer 188 were combined, a synergic effect was evidenced between the two agents (not shown). For example, the...
micin was combined with the poloxamer at 1% or 2% (w/v), the bacteria by of the infection without increasing the possibility of ototoxic and current brucellosis therapies, and could ensure efficient clearance 
encapsulated gentamicin achieved bacterial reduction at levels 
dervative burst of monocyte-macrophages. In this work, we hypothe-
ically, nevertheless, which the encapsulated drug reduces intracellular 
uptake to improve understanding of the mechanism and kinetics by 
the intracellular concentration of gentamicin after microsphere 
release from the microspheres was prolonged over up to 50 days, 
MBCs (3 log₁₀ reduction) of gentamicin and poloxamer 188 tested 
alone were 0.06 mg/L (poloxamer 1%) and 0.015 mg/L (poloxamer 2%).

Discussion

Our previous studies showed that gentamicin-containing PLA and PLGA microspheres are efficiently phagocytosed and that end-group uncapped PLGA 50:50H microspheres stimulated highly the ox-
idative burst of monocyte-macrophages. In this work, we hypothe-
sized that intracellular oxygen radicals produced upon microsphere 
internalization might act synergically with the antibiotic in killing 
intracellular bacteria.

Gentamicin-loaded PLGA 50:50H microspheres decreased 
significantly the intracellular bacterial levels (typically by 2 log₁₀) 
compared with untreated monocytes and free gentamicin; the effi-
ciency of PLGA 50:50 microspheres was lower (typically 1 log₁₀ 
reduction) and normally no different to the free drug; placebo micro-
ospheres had no effect. Opsomization of gentamicin-loaded micro-
spheres in fresh mouse serum did not improve the antibiotic efficiency of 
the treatment although, in previous work, opsomization increased 
the uptake of placebo microspheres and the cell oxidative meta-
bolism. The release of oxygen metabolites can alter the bacterial 
membrane permeability and thereby increase drug efficiency. 
Because Brucella spp. alone only slightly increase the oxidative burst 
in blood leucocytes, we speculated that an enhanced production of 
oxidative metabolites triggered by the phagocytosis of microspheres 
should improve treatment efficacy. This hypothesis was supported by 
the observation that brucellcidal activity of human polymorpho-
nuclear leucocyte granule lysates was enhanced by addition of hydro-
gen peroxide. In our experiments, no bactericidal effect was 
observed with placebo PLGA 50:50H microspheres. Nevertheless, 
the stimulation of oxygen derivatives within the cells may have 
increased the bacterial membrane permeability and made them more 
susceptible to the antibiotic.

Free gentamicin sulphate lowered the number of intracellular 
bacteria by −1 to 1.8 log₁₀. Because gentamicin cannot diffuse 
through cellular membranes, its internalization and accumulation 
inside cells has been ascribed to pinocytosis. The observed varia-
tion in the reduction in bacterial counts might result from variations 
in the pinocytic capacity of the cells. In our investigation, micro-
encapsulated gentamicin achieved bacterial reduction at levels 
superior or comparable to free drug. Considering that gentamicin 
release from the microspheres was prolonged over up to 50 days, 
antibiotic activity may be expected for prolonged periods of time. 
Incubation times were not prolonged in our studies due to limited cell 
viability and high bacterial proliferation. We hypothesize that this 
may provide intracellular bactericidal gentamicin concentrations over 
prolonged periods of time. Thus, this approach could reduce the 
multiple injections needed in the aminoglycoside component of 
current brucellosis therapies, and could ensure efficient clearance of 
the infection without increasing the possibility of ototoxic and 
nephrotoxic reactions. It would therefore be interesting to quantify 
the intracellular concentration of gentamicin after microsphere 
uptake to improve understanding of the mechanism and kinetics by 
which the encapsulated drug reduces intracellular Brucella. None-
theless, in vivo processing of the microspheres is likely to affect the 
kinetcis of drug release and polymer degradation. Intracellular reduc-
tion of Brucella was greater for PLGA 50:50H than for PLGA 50:50 
microspheres, although in vitro burst release within the initial 24 h of 
incubation amounted to only 14% for the PLGA 50:50H as compared 
with 50% for the PLGA 50:50 microspheres. Two factors may 
explain the results. First, the pronounced stimulation of oxidative 
metabolites by the PLGA 50:50H particles may have enhanced the 
antibiotic activity of gentamicin. Second, a higher number of PLGA 
50:50H particles might have been phagocytosed, because a larger 
amount of these particles had been added to the infected monocytes to 
achieve identical gentamicin doses (1 mg of PLGA 50:50H versus 
0.7 mg of PLGA 50:50 microspheres, with both types having a similar 
phagocytosis rate of ~50%). However, the present data are insuffi-
cient to conclude that a higher number of phagocytosed particles 
directly affect antimicrobial efficacy.

An interesting result of this study was the increased antibacterial 
activity (up to 1.2 log₁₀) of microencapsulated gentamicin when 
the dispersion medium for the microspheres contained 2% (w/v) 
poloxamer 188. Several mechanisms may be responsible for this 
phenomenon: (i) adsorption of the surfactant on the microsphere 
surface thereby altering the surface polarity and the potential for cell 
adhesion; (ii) enhanced phagocytosis due to a higher degree of par-
ticle dispersion or another mechanism; (iii) non-specific cell activa-
tion by the surfactant; and (iv) synergic enhancement of antibiotic 
activity. Particle coating by poloxamer type surfactants is well 
known to suppress phagocytosis in vitro and alter the opsonization by 
serum proteins in vivo, slowing down the elimination of particles 
from the blood by the mononuclear phagocytic system. The efficiency 
of poloxamer types to reduce particle phagocytosis depends 
on the molecular weight and chain length of the propylene oxide (PO) 
and ethylene oxide (EO) blocks. Thin coatings conferring poor 
steric stabilization were obtained on highly polar surfaces where 
the PO blocks were adsorbed preferentially onto the particle surface, 
wheras the EO chains stretched out into the dispersion medium. 
On larger particles, such a thin layer was probably adsorbed flatly so 
that the hydrophobic PO units remained exposed to the medium. 
With the relatively polar and hydrophilic PLGA 50:50 and PLGA 
50:50H microspheres studied herein, adsorption of poloxamer prob-
ably occurred through interaction with the EO chains, resulting in a 
flat arrangement of adsorbed poloxamer. As a result, the exposed PO 
chains possibly increased the hydrophobicity of the particle surface, 
leading to increased particle uptake by monocytes, as observed by 
optical microscopy (data not shown). Further, improved particle dis-

cipersion in 2% poloxamer 188 should have also contributed to the 
increased phagocytosis. Finally, mechanisms involving interaction 
between poloxamer and cell membrane or cell activation cannot 
be excluded. Some poloxamers have indeed increased phagocytic 
activity, and altered bacterial cell wall integrity and permeability 
yielding a synergic effect with antibiotic agents. Our suscepti-
bility studies in culture broth demonstrated that poloxamer can 
potentiate the antibacterial activity of gentamicin. Enhancement of 
antibiotic susceptibility was observed with subinhibitory concentra-
tions of poloxamer. However, no bactericidal effect of 2% soluble 
poloxamer alone or 2% poloxamer incubated with placebo micro-
ospheres was observed on the intracellular bacterial counts. The results 
indicate that only non-inhibitory amounts of poloxamer may reach 
the intracellular bacteria. Nevertheless, non-inhibitory concentra-
tions of the surfactant may have synergically enhanced the anti-
Brucella activity of gentamicin delivered intracellularly from micro-
spheres by as yet unknown mechanisms.

Finally, an important aspect for discussing the effectiveness of 
encapsulated gentamicin is the location of the Brucella inside the 
cell. Intracellular bacteria can evade the host defences by different
Microspheres to treat Brucella-infected macrocytes

mechanisms and prevention of phagosome–lysosome fusion has been proposed for intracellular survival of virulent Brucella spp., e.g. B. abortus 2308.27 B. abortus is located in the perinuclear region within compartments resembling autophagosomes and is then delivered to the endoplasmic reticulum where intracellular bacterial replication takes place.28–30 This special localization of B. abortus might prevent contact between bacteria and antibiotic-loaded microspheres. On the other hand, virulent Brucella can transit from early endosomes to autophagosomes, which apparently did not fuse with endosomes loaded with exogenous material.30 Nonetheless, fusion between phagosomes and other endocytic vesicles can generally occur, so that phagosomes loaded with particles or pinocytic endosomes have the potential to eliminate Brucella that reside in accessible phagosomes.17 Two localizations for B. abortus have been found at 24 h post-infection: accessible single membrane phagosomes and multimembranous autophagosomes,28 where the bacteria may escape from the antibiotics delivered to phagosomes.

In conclusion, we suggest that microsphere internalization is responsible for the significant reduction in the intracellular bacteria by gentamicin-loaded PLGA 50:50 and PLGA 50:50H microspheres within 24 h. Microencapsulated gentamicin was efficiently targeted to infected monocytes and reduced intracellular Brucella infections. In addition, gentamicin may still be available for further release from the microspheres for prolonged periods of time. Poloxamer 188 added to the dispersion medium for the microspheres significantly enhanced the efficiency of the antibiotic treatment. Further studies should focus on determining the mechanisms by which microencapsulated gentamicin is capable of reducing intracellular viable Brucella and investigate the efficacy of gentamicin-loaded microspheres in the treatment of brucellosis in vivo.

Acknowledgements

We would like to thank Peter Rigsby for his assistance in the statistical analysis and Dr Socorro Espuelas for helpful discussions. This work was supported by the Programme Redes Temáticas de Investigación Cooperativa del FIS—Brucellosis, ref. no. G03/201, and ‘Proyectos de Investigación Universidad de Navarra’ (PIUNA–2000–10) from the University of Navarra, Spain. Fellowship support for S.P. from ‘Asociación Amigos de la Universidad’ is gratefully acknowledged.

References


