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In vitro cell compatibility and antibacterial activity of microencapsulated doxycycline designed for improved localized therapy of septic arthritis

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Objectives: For the treatment of septic arthritis in large animals, the local application of antibiotics as a slow release system may be an appropriate means to reach high local bioactivity and low systemic side effects and drug residues. In this study, doxycycline microspheres were developed and tested in vitro for their drug-release properties, suitability for intra-articular application and antimicrobial activity.

Methods: The development of a slow release system was achieved by microencapsulation of the drug into poly(lactide-co-glycolide) microspheres by a novel ultrasonic atomization method. Drug elution was evaluated from microspheres dispersed in elution medium at pre-defined time points by HPLC. Joint-tissue compatibility was tested on cultured bovine synoviocytes by evaluating the expression of pro-inflammatory cytokine mRNA and the production of nitric oxide (NO). Finally, the antimicrobial activity of the released antibiotic was assessed with Gram-negative and Gram-positive bacteria exposed to release medium sampled at days 1, 7 and 12 after microsphere suspension.

Results: An adequate size of the microspheres, sufficient stabilization of doxycycline in aqueous environment and drug release (25 mg microspheres in 4 mL medium) above MIC for bacteria usually isolated in bovine and equine joints were obtained over 15 days. Although the cytokine mRNA expression reflected the excellent tissue compatibility, the results with NO yielded contradictory results. Antimicrobial tests of the release medium proved to match perfectly the activity of non-encapsulated, free doxycycline as reported in the literature.

Conclusions: The newly developed doxycycline delivery system achieved the target specifications and is ready for in vivo testing.

Keywords: septic arthritis, microspheres, in vitro toxicity, antimicrobial activity

Introduction

Treatment of septic arthritis in large animals is demanding and prognosis still guarded.1–3 Besides surgical interventions, the immediate onset of antibiotic therapy is mandatory.4 Local application of antibiotics has the advantage of providing high drug concentrations at the site of infection while keeping the systemic concentrations low, which results in less systemic side effects and lower drug residues in muscular tissues.5,6 Disadvantages of local drug application are the risk of joint tissue toxicity and superinfections following repeated arthrocenteses.4,7

Attempts to overcome the problems of repeated local drug administration have primarily focused on using slow release formulations of antibiotics.8 Two slow release antibiotic products are commercially available for human treatment in Switzerland.
Doxycycline microspheres tested for septic arthritis therapy

The first consists of gentamicin-containing poly(methylmethacrylate) (PMMA) beads (Septopal®-Ketten, Biomet Orthopaedics, Ried b.Kerzers, Switzerland), which can be implanted into infected wounds, septic joints and in the vicinity of fractured bones. After healing or exhaustion of the antibiotic activity, the beads have to be removed surgically.3,9-11 The second marketed formulation is a gentamicin-soaked sponge made from bovine collagen (Garamycin® Schwann, Essex Chemie, Luzern, Switzerland). The collagen sponge is biodegradable and does, therefore, not require a second surgical intervention. Furthermore, it diminishes the risk of developing a resistant flora as described for the PMMA beads.3,12,13 Conversely, the collagen sponge affords shorter periods of drug release than the PMMA beads14,15 and possesses the risk of propagating transmissible spongiform encephalitis.

Investigational studies have considered a variety of antibiotics and slow release formulations, many of which were based on PMMA or on biodegradable poly(lactide) or poly(lactide-co-glycolide) (PLGA).16-19 The most commonly used antibiotic in these studies was gentamicin. Gentamicin covers a broad antibiotic spectrum including bacterial pathogens often isolated from equine joints. However, it is also known for its irritating activity by inducing a mild and transient synovitis7 and its relative inefficiency against anaerobic bacteria often isolated from bovine joints.20-22 A good alternative to gentamicin for slow release formulations designed for the treatment of infected joints is doxycycline hyclate. Introduced into human pharmacotherapy in 1967,23 it has proven its antibiotic efficacy against most of the pathogenic bacteria isolated from inflamed joints of horses and cattle.23-25 However, resistance to tetracyclines is a worldwide problem. It is due to either decreased intracellular antibiotic accumulation caused by inhibition of drug import into the cell or increased antibiotic efflux, or to a mechanism to protect cellular ribosomes. Generally, there is cross-bacterial resistance among tetracycline and doxycycline, but there are several exceptions described (e.g. Bacteroides fragilis26 and Streptococcus pneumoniae27). When focusing on bacteria relevant for septic arthritis, a general overview about the susceptibility of bacterial isolates from horses in The Netherlands24 reports some resistance of Escherichia coli and Klebsiella spp., but none of the tested Streptococci and Staphylococci spp. and Actinobacillus equi showed MICs above 2 mg/L. All Staphylococcus aureus isolates from cattle in Brazil were also susceptible to doxycycline, whereas 7 of 46 strains were resistant to tetracycline.25

Doxycycline has been reported to be chondroprotective both in vitro28-30 and in human clinical studies.31,32 Our own experiments revealed a very good local tissue compatibility of doxycycline in bovine joints.33

Further critical issues relevant for developing and using slow release antibiotic formulations are the stability of the antibiotic in the formulation during the entire release period and the ease of localized administration, e.g. in infected joints of animals. Readily administrable slow release formulations are, for example, biodegradable microspheres made of PLGA. PLGA microspheres can be administered with a conventional syringe and needle. Several hormone-containing PLGA products are on the market for human use to systemically treat different forms of cancer. When considering the stability issue, many antibiotics will not be useful for slow release as they degrade quickly, i.e. within a few hours or days. Examples of unstable antibiotics include erythromycin, penicillins and cephalosporins, which hydrolyse quickly upon contact with aqueous media. Tetracyclines, although not susceptible to hydrolysis, readily undergo oxidation and epimerization. Nevertheless, doxycycline is one of the most stable tetracycline group members and has, in our preliminary experiments, promised to be a good candidate for slow release formulations.34 The feasibility of microencapsulating tetracycline compounds into PLGA has been demonstrated for minocycline used to treat periodontitis in man.35

In the present study, we developed slow release PLGA microspheres for the delivery of doxycycline as improved treatment of infected joints of horses and cattle. The formulations were tested for the duration of doxycycline release, doxycycline stability, in vitro toxicity and in vitro antimicrobial activity.

Materials and methods

Doxycycline microencapsulation into PLGA microspheres

Doxycycline hyclate (Ph. Eur.; Spirig, Egerkingen, Switzerland) was microencapsulated into PLGA 50:50 (PLGA 50:50, Resomer® 502 H, Boehringer-Ingelheim, Ingelheim, Germany) at 10% nominal drug loading. During processing and storage, all materials were protected from light and oxygen. For a 3 g batch, 300 mg doxycycline was dissolved in 3 mL of methanol, and this solution was added slowly to a solution of 2.7 g of PLGA dissolved in 27 mL of acetone. The clear and slightly yellowish doxycycline/PLGA solution was atomized at a rate of 30 mL/h through an ultrasonic spray-head (US1, Lecher, Metzingen, Germany) into 500 mL of octamethylcyclotetrasiloxane (OMCTS; Abil K-4, Goldschmidt, Essen, Germany) under moderate stirring. At the end of atomization, the slurry was exposed to a slightly reduced pressure (400 mbar) for 3 h to eliminate further the solvents and harden the microspheres. The microsphere dispersion in OMCTS was filtered through a 0.2 μm regenerated cellulose filter (Schleicher & Schuell, Dassel, Germany), washed three times with 20 mL of cyclohexane and dried overnight at 30°C and 20 mbar. For storage, the microspheres were filled into brown glass vials, purged with argon and kept at 4°C in the dark. A portion of the microspheres was also subjected to γ-irradiation (25 kGy, Studer Hard, Dübendorf, Switzerland) to test potential instability and degradation during sterilization.

Determination of microsphere size and doxycycline loading

Particle size was analysed by laser light diffraction (Mastersizer X, Malvern, UK). The doxycycline content in the microspheres was determined by dissolving 10 mg of microspheres (accurately weighed) in 8.0 mL of acetonitrile and completing the solution with 2.0 mL of water. The filtered solution (0.45 μm cellulose acetate filter) was analysed by HPLC.36 using an EZCHROM Elite Hitachi (Hitachi, VWR International, Dietikon, Switzerland) with a UV-VIS detector L-2420, an autosampler L-2200 and a pump L-2130. Separation was performed on a C8-Phenomenex Luna column (5 μm, 250 x 4.6 mm; Brechbühler, Schlieren, Switzerland) under isocratic conditions using as mobile phase a degassed mixture of acetonitrile/water/perchloric acid (26:74:0.25, by vol.) adjusted to pH 2.5 with...
In vitro doxycycline release testing

Microspheres (20 mg, accurately weighed) were filled in 4.5 mL amber borosilicate screw-top vials (BGB Analytik, Adliswil, Switzerland) and dispersed in 4.0 mL of release medium. The release medium consisted of a 0.1 M citrate buffer of pH 5.0, supplemented with 0.01% (w/w) sodium azide, 0.01% (w/w) poloxamer 188 and 0.01% sodium EDTA. The vials were installed in a drum rotating at 3 rpm and placed at 37°C. At regular time intervals, the entire release medium of four vials was withdrawn and analysed by HPLC, as described in the previous section. The remaining polymeric pellets were dried under vacuum overnight, and the non-released doxycycline extracted and analysed by HPLC as described in the previous section.

In vitro toxicity testing of doxycycline and doxycycline-loaded microspheres on cultured bovine synoviocytes

Establishment of bovine synovial cell cultures. Synovial membranes of four calves and two cows (aged between 6 months and 4 years), who were routinely slaughtered in a local abattoir, were harvested aseptically from fetlock (metacarpo/tarso-phalangeal) joint. A separate culture was prepared for each animal. After two washes in saline, the membranes were cut into small pieces of 1 mm², placed into the inner part of a two-compartment digestion chamber, pre-treated with 0.2% trypsin at 37°C for 30 min and subsequently digested enzymatically with 0.2% collagenase for 2 h at 37°C under constant movement. The cells were collected from the outer compartment of the digestion chamber, washed three times with Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen, Basel, Switzerland) containing 10% fetal bovine serum and subsequently seeded in six-well plates (Falcon Companion Plate 6 Wells, Milian, Meyrin, Switzerland) at 100 000 cells per well. For maintenance, the cells were cultured at 95% humidity, 5% CO₂ and 37°C for 7–8 days until confluence, with the medium being changed every 2–3 days.

Cytotoxicity testing. Preliminary tests were performed to evaluate the cytotoxicity of bacterial lipopolysaccharide (LPS) using different concentrations and incubating over different periods of time. Incubation time and concentrations were chosen to prevent enhanced cell death. Cell numbers were assessed using a cell viability test based on cleavage of tetrazolium salt (Cell Proliferation Reagent WST-1, Roche Diagnostics, Mannheim Germany). β-Actin-messenger RNA levels proved to correlate significantly (P < 0.001) with the viability test and were thus chosen for normalization.

For toxicity experiments, the cells were vigorously washed with phosphate-buffered saline (PBS) containing 0.2 g/L KCl, 1.0 g/L NaHCO₃ and 1 g/L glucose monohydrate and treated with (i) plain DMEM (negative control), (ii) 100 mg/L LPS (Sigma-Aldrich, Buchs SG, Switzerland) (positive control), (iii) 1 mg/mL doxycycline in solution (Vibravenoes®, Pfizer, Zurich Switzerland), (iv) 0.2 mg/mL microspheres (low-dose microspheres; 20 μg/mL doxycycline), and (v) 5 mg/mL microspheres (high-dose microspheres; 500 μg/mL doxycycline). For incubation, all test materials (LPS, doxycycline, and microspheres) were dissolved or suspended in DMEM; incubation lasted for 4 or 24 h. With the high-dose microspheres, the cells were completely and firmly covered with the microspheres, which compromised the further separation and lysis of the cells. Hence, an insert (Falcon 0.4 mm HD Inserts, Milian) was used for the high-dose microspheres to allow separation of microspheres and cells and to facilitate the collection of the cells. The supernatant of all cell samples was collected and kept at −80°C until further use. The cells were lysed in TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA), their RNA extracted following the manufacturer’s instructions and the extracts kept at −80°C until further processing. Each test was performed in triplicate.

The differently treated synoviocytes were analysed for the production of interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α mRNA by RT–PCR. β-Actin was used as the housekeeping gene. The primers and probes (Table 1; all from Microsynth, Balgach, Switzerland) were designed on the basis of the GenBank database and with the help of the Primer Express Oligo Design software version 1.5 (Applied Biosystems, Foster City, CA, USA). The probes were designed to contain an exon–exon junction overlapping sequence to exclude the amplification of genomic DNA and they were labelled with the reporter molecule 6-carboxyfluorescein (FAM) at the 5' ends and with the quencher molecule 6-carboxy-N,N',N'-tetramethylrhodamine (TAMRA) at the 3' ends. As a reference, cDNA from stimulated bovine macrophages (kindly provided by Prof. T. Jungi, Institute of Immunology, Vetsuisse Faculty, University of Berne, Berne, Switzerland) was used for assessing the efficiency of multiplication and calculating the mRNA content (eff⁻ⁿ⁻). Reverse transcription of 1 μg of RNA per cell sample was performed with TaqMan reverse transcription reagents (Applied Biosystems) using random hexamer primers. PCR was carried

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
<th>Efficiency</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>5'-TCA CTA CAG TGA CGA GAA TGA GCT GTT</td>
<td>5'-GGT CCA GGT GTT GGA TGC A</td>
<td>TCA TCA GCC TCA A</td>
<td>1.724</td>
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<tr>
<td>IL-6</td>
<td>5'-ACT GGC AGA AAA TAA GCT GAA TCT TC</td>
<td>5'-TGA TCA AGC AAA TCG CCT GAT</td>
<td>TCC TCC TTT</td>
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<tr>
<td>TNF</td>
<td>5'-CCT CTT CTC AAG CCT CAA GTA ACA A</td>
<td>5'-GAG CTG CCC CGG AGA GTT</td>
<td>5'-ATG TCG GCT ACA ACG TGG</td>
<td>1.84</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CGG CAC CAG GGC GTA AT</td>
<td>5'-TCT CCA TGT CGT CCC AGT TG</td>
<td>5'-AGC AAG AGA GGC ATC CTG ACC CTC AAG T</td>
<td>1.79</td>
</tr>
</tbody>
</table>

Table 1. Primers and probes used for real-time RT–PCR of cytokines and the corresponding multiplication efficiency factors
out in 24 µL reactions with 900 nM of each primer, 250 nM of labelled probe and Universal PCR master mix (Applied Biosystems) under standard thermal conditions with an AB 7500 Real Time PCR System (Applied Biosystems).

The nitric oxide (NO) release from the synoviocytes incubated during 24 h with doxycycline solution, doxycycline-loaded microspheres and control samples was examined using the Total NO/Nitrite/Nitrate Assay (R&D Systems, Bühlmann Laboratories, Schönernbuch, Switzerland), which is based on the Griess reaction. The amounts of NO were normalized to the amount of β-actin mRNA.

Contents of mRNA and NO were expressed relative to the amounts of the negative control (DMEM) at 4 h (mRNA) and 24 h (NO). Statistical data analysis was performed using the Number Cruncher Statistical System (Kaysville, UT, USA). Groups were compared using one-way analysis of variance (ANOVA), and the P value was set at 0.05.

**In vitro antimicrobial activity testing of doxycycline released from microspheres**

Release test medium from six samples, each of non-irradiated and γ-irradiated doxycycline-loaded microsphere preparations (see the section on Doxycycline microencapsulation into PLGA microspheres), was used for the assessment of the antimicrobial activity of doxycycline released after 1, 7 and 12 days. The day before the sampling of the release test medium for microbial testing, the entire release medium was discarded and replaced by fresh release medium (see the section on In vitro doxycycline release testing; medium without sodium azide). This procedure eliminated any doxycycline-degradation products developing in the release medium over time and considered only the amount of doxycycline released within 24 h at the above indicated time points. Samples were stored at −20°C until further use. Before performing the antibacterial tests, the doxycycline concentration was assessed for every sample with the HPLC analysis (see the section on Determination of microsphere size and doxycycline loading).

The in vitro antibacterial activity of the released doxycycline against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 was determined using the microdilution method in cation-adjusted Mueller–Hinton broth (CAMBH), according to the CLSI guidelines. Doxycycline hyclate solutions in citrate buffer (concentrations from 64 to 0.125 mg/L) served as reference dilutions and plain elution buffer as negative control. Release test samples from microspheres were pre-diluted or used undiluted according to the results from HPLC measurements. Every sample was subjected to serial dilutions in order to evaluate the individual MIC as calculated from the number of dilution steps. Bacterial cultures were expanded in CAMBH at 37°C for 4–6 h. Inocula were adjusted to 1 × 10^6 bacteria/mL nephelometrically, using standardization curves determined in earlier studies. Microtitre plates (U-shaped bottom; Greiner, Frickenhausen, Germany) were loaded with 50 µL of serially diluted release testing samples and controls. To each well, 50 µL of standard inoculum was added and incubated for 24 h. The MIC was read as the lowest concentration without visible growth.

**Statistical analysis**

The data were statistically analysed by ANOVA and Bonferroni post hoc test. P values less than 0.05 were considered significant.

**Results**

**Yield, size and doxycycline content of the microspheres**

The product yield was in the range of 70% to 75%, and the volume weighted particle size range was between 15 and 120 µm with a mean diameter of 35 µm. The doxycycline content in the non-irradiated and γ-irradiated microspheres amounted to 82 and 77 µg/mg microspheres, respectively, corresponding to an encapsulation efficiency of more than 80%. No differences were observed in the HPLC chromatograms between non-irradiated and irradiated samples in terms of additional peaks, thus excluding potential doxycycline degradation.

**Figure 1.** Doxycycline release profiles from non-irradiated (open circles) and γ-irradiated (25 kGy) (filled circles) PLGA 50:50 microspheres. (a) Doxycycline release directly measured in the test medium. (b) Doxycycline release determined as the difference between the primary doxycycline content of microspheres and the amount of non-released doxycycline that remained in the polymeric pellet at the indicated time points.
products. Therefore, in microencapsulated form, doxycycline appeared to remain stable during γ-irradiation.

**In vitro release of doxycycline**

To assess the stability of doxycycline at 37°C over prolonged periods of time, the amount of released doxycycline was assessed in two ways: by analysing doxycycline in the release medium (Figure 1a) and by determining the amount of non-released doxycycline in the remaining polymeric mass (Figure 1b). Both analytical methods revealed a triphasic release profile, lasting ~15 days, and no significant differences were found between non-irradiated and γ-irradiated microspheres (Figure 1).

When doxycycline was analysed in the release medium, the triphasic release profile consisted of an initial burst amounting to ~30 μg/mg microspheres, a period of very little doxycycline release that lasted ~3 days, and a final phase of almost constant release of an additional ~30 μg/mg microspheres between days 4 and 15 (Figure 1a). After day 15, a slight decrease suggested some degradation of doxycycline in the release medium, which was confirmed by the appearance of an additional elution peak in the HPLC chromatograms (R. Alder and A. Pickl, data not shown). Thus, ~20 μg doxycycline/mg microspheres degraded in the release medium within 15 days of incubation.

Calculating from the amount of non-released doxycycline in the remaining polymeric mass, both the burst (~58 μg/mg microspheres) and total amount released (~98 μg/mg microspheres) were higher (Figure 1b), and there was no apparent loss of material after completion on day 15. The apparently excessive total amount of doxycycline released (~98 μg/mg microspheres versus 82 μg/mg microspheres doxycycline loading) must be ascribed to small yet cumulative quantities of doxycycline remaining in the vials (remaining release medium, vial wall, adsorbed on the surface of polymer pellet) each time the release medium was withdrawn; these residual amounts of doxycycline in the vial were added to the amount of non-released doxycycline, as the remaining polymeric material was dissolved in the vial itself.

**In vitro toxicity of doxycycline and doxycycline-loaded microspheres on cultured bovine synoviocytes**

The synoviocytes incubated for 4 h with doxycycline or doxycycline-loaded microspheres expressed similar amounts of cytokine-specific mRNA to those incubated with plain medium, but significantly less than the positive control cells stimulated with LPS (P < 0.01) (Figure 2). When normalizing the mRNA levels of the treated cells against the negative control cells (incubated with plain medium), the IL-1β-specific mRNA was increased by factors of 1.2 (plain doxycycline), 1.5 (low-dose microspheres), 1.8 (high-dose microspheres) and 11.0 (positive LPS control) (Figure 2a). For the IL-6-specific mRNA (Figure 2b), the relative changes of expression level over that observed with the negative control cells were 3.4 with plain doxycycline, 1.0 with the low-dose microspheres, 0.7 with the high-dose microspheres and 33.2 with the LPS control. For the TNF-α-specific mRNA (Figure 2c), the relative changes were 2.0 with the plain doxycycline, 1.8 with the low-dose microspheres, 2.9 with the high-dose microspheres and 39.3 with the positive control.

After 24 h of incubation, the mRNA expression levels changed variably depending on the treatment. When compared with the negative control at 24 h, the IL-1β-specific mRNA (C. Haerdi-Landerer, data not shown) did not change with plain doxycycline, but increased by a factor of 29.0 in the positive control and decreased by factors of 0.7 and 0.1 with the low-dose and high-dose microspheres, respectively. For the non-stimulated cells, the increase in IL-1β mRNA expression between 4 and 24 h was 1.3-fold. The IL-6 mRNA (C. Haerdi-Landerer, data not shown) increased by factors of 13.1 (plain doxycycline), 239.8 (positive control) and 1.9 (high-dose microspheres), but decreased slightly (by a factor of 0.9) with the low-dose microspheres when compared with the negative control at 24 h. For the negative control cells, the IL-6 mRNA expression decreased by the factor of 0.34 between 4 and 24 h. Finally, the TNF-α mRNA levels (Figure 3) at 24 h were increased by factors of 10.4 (positive control), 3.6 (plain doxycycline), 1.03 (low-dose microspheres), and 7.8 (high-dose microspheres).
**Doxycycline microspheres tested for septic arthritis therapy**

In vitro antimicrobial activity of doxycycline released from microspheres

HPLC measurements of doxycycline in release media of microspheres revealed mean values of 200.5 ± 8.2 mg/L for day 1, 14.1 ± 5.2 mg/L for day 7 and 22.4 ± 1.1 mg/L for day 12. Thus, day 1 media were diluted 1:9 before starting serial dilution. With *E. coli* ATCC 25922, ranges of MICs for doxycycline in the release medium were 0.6–1.26 mg/L for day 1 samples, 0.41–0.92 mg/L for day 7 samples and 0.66–0.75 mg/L for day 12 samples. With *S. aureus* ATCC 25923, the MIC value ranges were 0.15–0.32 mg/L for day 1, 0.15–0.46 mg/L for day 7 and 0.16–0.19 mg/L for day 12. Thus, the MIC values of released doxycycline against both bacterial strains were kept within the limits in terms of strain-specific CLSI doxycycline reference MIC values (*E. coli*: 0.5–2.0 and *S. aureus*: 0.12–0.5). No differences were found between the three different time points or between samples from γ-irradiated and non-irradiated microspheres. Overall, the antimicrobial activity of doxycycline released from microspheres after 1, 7 and 12 days generally coincided with the original substance.

**Discussion**

The aim of this study was to produce doxycycline-containing microspheres at high yield, with good encapsulation efficiency, particle sizes above the threshold for uptake by phagocytes, controlled antibiotic release over ~14 days, low inflammatory reaction and intact antibiotic activity. Further, the controlled release and bioactivity of the formulation should remain unaltered upon sterilization by γ-irradiation. These specifications, defined in view of subsequently using the delivery system for the localized and prolonged treatment of septic arthritis in large animals, were all successfully met in this study.

The microencapsulation of antibiotics generally faces some of the following hurdles: drug degradation, modest encapsulation efficiency, alteration by sterilization or scaling-up difficulties. Different methods for microencapsulation of antibiotics have been described.\(^{35,39,40}\) Spray-drying was excluded, because it is known to yield, at least with laboratory spray-dryers, small particles (diameters of 0.1–10 μm) that are highly susceptible to phagocytosis, especially in inflamed tissue. The solvent extraction/evaporation methods generally yield modest encapsulation efficiency with highly water-soluble drugs, besides being difficult to scale up. Indeed, our own preliminary experiments confirmed a very low encapsulation efficiency of doxycycline in PLGA 50:50 microspheres when using a microextrusion-based solvent extraction method (S. Fischer and B. Gander, unpublished results). The method developed in this study was tailored to the particular materials used and the pre-defined aforementioned specifications. The ultrasonic atomization of the organic doxycycline–PLGA solution into the non-solvent OMCTS...
produced the desired particle size range at a high yield. The absence of water in the process was most favourable for the high encapsulation efficiency and preservation of the doxycycline stability. Furthermore, the method can be readily scaled-up and transferred to an industrial setting.

Drug stability is definitely the first prerequisite for prolonged release over the desired time interval of 10–15 days. Many antibiotics such as penicillins, cephalosporins and erythromycin are sensitive to humidity and hydrolyse in the presence of water. These drugs are obviously unsuitable for prolonged release from PLGA microspheres, which release entrapped drugs by diffusion upon hydration of the particles in water and hydrolytic degradation of the polyester. Our own attempts to microencapsulate a cephalosporin compound into PLGA failed badly, because we were unable to slow down sufficiently the hydrolysis of the drug (S. Fischer and B. Gander, unpublished results). For the intended therapeutic use, a good alternative to cephalosporins are the tetracyclines. Tetracycline and its analogues do not undergo hydrolysis, but are susceptible to photolysis, epimerization and isomerization.\textsuperscript{34,36} Doxycycline hyclate was selected for our work, because it is one of the most stable tetracycline analogues and it is already successfully used in drug delivery systems (DOXY\textsuperscript{®}, Vivadent Ivoclar, Schaan, Liechtenstein; Atridox\textsuperscript{®}, CollaGenex Pharmaceuticals, Newtown, PA, USA) for periodontal application. For developing a suitable in vitro release test medium for the doxycycline-loaded microspheres, preliminary stability experiments were conducted in different buffer systems and by the addition of putative stabilizers. Here, an enhanced doxycycline stability was noticed in pure water and citrate buffer of pH 5.0 over PBS of pH 7.4 (20\% versus 30\% degradation within 7 days at 37 °C). A further improvement was achieved by adding sodium EDTA (0.01\%) to the citrate buffer (12\% degradation within 7 days at 37 °C) (A. Pickl and B. Gander, data not shown).

The in vitro doxycycline release from the PLGA 50:50 microspheres in the optimized medium lasted for 14 days. The initial burst was expected for the highly water-soluble antibiotic and should be appropriate to reach an immediate biologically active antibiotic concentration. The apparent total release was, however, substantially lower when doxycycline was analysed in the release medium when estimated from the amount of non-released drug (Figure 1a versus 1b). This demonstrates good protection of doxycycline within the PLGA microspheres against epimerization and oxidation. Furthermore, γ-irradiation did not significantly degrade doxycycline within the microspheres or alter the in vitro release profile. This negligible effect is especially important in view of developing a product for parenteral application; it was, however, surprising because many, if not most, of the published studies on the γ-irradiation of PLGA-encapsulated drugs reveal significant drug degradation and accelerated drug release after irradiation of the microspheres.\textsuperscript{31,42} We may speculate that the absence of water during microencapsulation yielded a product with very low residual humidity. Therefore, the amount of H\textsuperscript{•} and OH\textsuperscript{•} radicals formed during irradiation may have been extremely low, thus limiting substantially any subsequent radiolysis of drug or polymer. Importantly, the feasibility of γ-irradiation of the doxycycline-loaded microspheres facilitates greatly the production of controlled release doxycycline for improving the therapy of septic arthritis in large animals.

As an endeavour to reduce animal tests, cultured synoviocytes from several species have been used for tissue compatibility testing of drugs.\textsuperscript{43,44} In joint tissues, inflammation and matrix degradation have been strongly associated with increased levels of the cytokines IL-1β, IL-6 and TNF-α. Thus, these markers are often used as parameters for the evaluation of joint irritation and inflammation.\textsuperscript{43,45,46} In our study, we noticed significant differences of cytokine mRNA expression in bovine synovial cells treated with and without bacterial LPS, which demonstrates the suitability of this test system for evaluating the in vitro cell compatibility of the developed delivery system. The minimal changes of cytokine expression observed with the doxycycline-loaded microspheres, when compared with the negative control, suggest a very good tissue compatibility of the microsphere formulation. The apparent impact of the duration of incubation on the TNF-α expression was surprising. A nearly 4-fold increase in TNF-α mRNA expression was observed from 4 to 24 h of incubation in the negative control cells. This must be assigned to the absence of fetal bovine serum. Furthermore, the starved cells were less tolerant to the exposure to plain doxycycline or the high-dose microspheres.

Another marker of tissue irritation and inflammation is NO. NO production by cultured synoviocytes has been used as a parameter for tissue irritation in horses,\textsuperscript{47} humans\textsuperscript{48} and rabbits.\textsuperscript{49} However, regulation of NO synthases differs between different species and also between different tissues.\textsuperscript{50,51} Nevertheless, the increased NO content in the supernatant of the cells stimulated with the doxycycline-containing microspheres has to be taken into account when interpreting the overall reaction of cultured synoviocytes to the delivery system. NO has been reported to act as inflammatory mediator, but its action within the cascade is contradictory, exerting protective as well as pro-inflammatory and catabolic activities on the cartilaginous matrix.\textsuperscript{52,53} Therefore, conclusive answers will only be obtained through in vivo experiments.

The antimicrobial tests proved the biological activity of the doxycycline released from both the non-irradiated and γ-irradiated microspheres. The MICs of the doxycycline released from the microspheres conformed exactly to the values reported in the literature, i.e. 0.5–2.0 mg/L for E. coli and 0.12–0.5 mg/L for S. aureus.\textsuperscript{38}

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Transparency declarations

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