In vitro/in vivo evaluation of the efficiency of teicoplanin-loaded biodegradable microparticles formulated for implantation to infected bone defects

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Chronic osteomyelitis is still the cause of many problems in orthopaedics in terms of therapy and infection persistence. Four-to-six week systemic antibiotic therapy is required along with bone and soft tissue debridement in the therapy of chronic osteomyelitis. Prolonged-release local antibiotic therapy has been taken into consideration due to the side effects encountered in long-term high dose antibiotic use and the duration of hospitalization of the patients. Although local antibiotic therapy has been achieved by bone cement, a second surgical operation is needed for the removal of the system. On the other hand, heat generation during cement curing limits the use of heat-sensitive active ingredients. The most frequent osteomyelitis inducing micro-organism is gram (+) Staphylococcus aureus. In this study, teicoplanin, a glycopeptide antibiotic, active on gram (+) bacteria, was incorporated in a synthetic polymer in order to prepare a microsphere formulation for implantation to bone defects. Particle size, surface characteristics, loading capacity and in vitro release characteristics of the microspheres were determined as well as stability assessment of teicoplanin under accelerated conditions. In vivo studies were performed on rabbits and the microparticles were implanted intra-articularly to the lateral condylus of the femur. Antibiotic presence was detected by a microbiological assay from synovial fluid sample aspirated throughout 5 weeks. In the light of these evaluations, microspheres prepared from PLGA (75:25) (Mw 136 000) polymer were determined to be effective, and promising for obtaining prolonged local antibiotic release.

Keywords: Implant, PLGA microspheres, teicoplanin, microbiological assay, osteomyelitis.

Introduction

A major development in recent years has been the fabrication of implantable drug delivery systems using biocompatible and biodegradable polymers (Danckwerts and

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Polymeric compounds have been widely used in designing the implantable controlled release delivery systems due to the various advantages they offer such as predictability of drug release profile and ease of fabrication (Danckwerts and Fassihi 1991, Shah et al. 1993).

Implants are generally administered by injecting subcutaneously by minor surgery or by the placement of a surgeon directly to or near the site required (Danckwerts and Fassihi 1991). In the case of non-biodegradable polymers, an implanted dosage form has to be removed at the end of the release period while the use of biodegradable polymers avoid a second surgical intervention (Danckwerts and Fassihi 1991, Shah et al. 1993).

In orthopaedics, biodegradable polymers are frequently used to produce fixation rods, plates, screws, suture anchors, and sutures (Athanasiou et al. 1995, 1998). Investigational uses of these materials include augmenting repair of musculoskeletal tissues, either as scaffolds for tissue in growth or carriers of tissues, cells or bioactive agents. Ongoing research in this field strongly suggests that these materials will also be used more extensively as fillers for bone defects and scaffolds for cartilage and meniscal repair (Athanasiou et al. 1998).

Osteomyelitis is a bone disease caused by a bacterial infection of the bone medullar cavity, cortex and/or periosteum (Dickie 1986, Dirschl and Almekinders 1993). It still remains an important and discouraging orthopaedic and clinical problem. Conventional treatment using systemic antibiotics is expensive, prone to complications and often unsuccessful. Moreover, systemic administration of antibiotics is an inherently inefficient method for achieving high local tissue drug concentrations, only a small fraction of a given dose reaches the site of infection. High systemic levels of antibiotics also imply the risk of organ toxicity. Delivering an effective antimicrobial at sufficiently high concentrations to the area of infection in combination with surgery is a recognized treatment for bone infection (Sampath et al. 1992, Lin et al. 1999). Recently, spherical non-biodegradable implants usually performed with polymethylmethacrylate (PMMA) bone cement beads in combination with standard treatments have been reported (Popham et al. 1991, Adams et al. 1992). The disadvantages associated with the PMMA beads include a second surgery to remove the beads and incomplete and very slow drug release (Adams et al. 1992, Sampath et al. 1992). More recently, a number of authors have attempted to incorporate antibiotics in biodegradable materials and ceramic composites. However, ceramic materials are generally very brittle to handle (Hollinger and Battistone 1986). Microparticulate delivery systems formulated using biodegradable polymers have advantages over conventional polymethylmethacrylate beads and intravenous antibiotics in several ways and seem to be promising for the localized treatment of osteomyelitis (Sampath et al. 1992, Schmidt et al. 1995, Lin et al. 1999, Liu et al. 1999). They provide bactericidal concentrations of antibiotics for the prolonged time needed to treat the particular orthopaedic infection. Additionally, variable biodegradability from weeks to years may allow to adjust release properties. As the system is biodegradable, there is no need for surgical removal. Because the biodegradable system dissolves slowly, the soft tissue or bone defect slowly reconstitutes (Liu et al. 1999).

Teicoplanin is a new glycopeptide antibiotic providing both in vitro and clinical efficiency against most gram positive organisms including Staphylococcus.
aureus, Staphylococcus epidermidis and Cornyobacterium ssp in patients with a variety of moderate and severe infections, especially osteomyelitis. Teicoplanin does not seem to be ototoxic and appears less nephrotoxic and is well tolerated in humans and animals (Coroneli 1987, Schmidt et al. 1995. Yenice and Çalış 2001).

The aim of this study was the development of a biodegradable microparticle system having controlled release characteristics for implantation to bone defects for the localized treatment of osteomyelitis. For this purpose, synthetic PLGA was used in which teicoplanin was incorporated. The bioactivity of the antibiotic was followed by a microbiological assay throughout the in vitro experimental period and antibiotic seemed to be preserved stable.

Materials and methods

Materials

Teicoplanin was a gift from Hoechst Marion Roussel (Istanbul, Turkey), poly-lactide-co-glycolide (PLGA) (75:25) supplied by Medisorb (Cincinnati, USA), polyvinyl alcohol (PVA) Sigma (USA), chitosan-H Dainishiseika (Japan), sodium oleate (SO), Aldrich (Germany), Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were obtained from Difco Laboratories (Detroit, MI, USA). Dimethyl sulphoxide was supplied by Merck (Darmstadt, Germany), Ketamine hydrochloride from Eczacibaşı (Istanbul, Turkey) and Xylazine hydrochloride from Bayer (Istanbul, Turkey). White New Zealand rabbits were raised locally.

Methods

Microbiological assay of teicoplanin. For the microbiological assessment of teicoplanin and stability evaluation purposes, broth microdilution and agar diffusion methods were used which still have a widespread use for the assay of antibiotics (Lewis et al. 1988, Awni et al. 1991, National Committee for Clinical Laboratory Standards 1997, Atkins et al. 1998, Cardoso and Schapoval 2000, Prior et al. 2000, Yenice and Çalış 2001).

- **Broth microdilution method**: The procedures recommended by the National Committee for Clinical Laboratories (NCCLS) were followed for determining Minimal Inhibitory Concentrations (MIC). All studies were carried out in Mueller Hinton Broth while *Staphylococcus aureus* ATCC 25923 was used as the test organism (National Committee for Clinical Laboratory Standards 1997).

- **Agar diffusion method**: Growth inhibition zones of teicoplanin were determined in the samples and Mueller Hinton agar was used throughout these experiments. *Staphylococcus aureus* ATCC 25923 strain was adjusted to \(10^8\) cfu/ml in the MHB-MHA, cooled to 45–50°C, mixed with bacterial suspension and then poured to 9 mm sterile petri dishes. Samples were pipetted into previously prepared 5 mm diameter wells in the inoculated agar plates. After incubation at 35°C for 24 h, the diameter of growth inhibition zones were measured in millimeters.
Validation of microbiological assay. Validations of the method used were realized by determination of linearity, precision, sensitivity and specificity of variation coefficients (USP XXIII 1995, Cardoso and Schapoval 2000). In the microbiological assay studies, zone inhibitions were determined using a vernier caliper having enough precision. A standard curve was constructed by plotting zone sizes vs log_{10} teicoplanin concentrations. Unknown concentrations were calculated from the curves derived from the standards present on the same plate.

Stability studies. The bioactivity of teicoplanin was followed for 6 months by a microbiological assay. On study day 0, 1280 μg/mL of teicoplanin solution was added to the flask under aseptic conditions in a laminar-cabin. During the accelerated stability studies, 40 ± 2°C temperature and 75 ± 5% relative humidity were maintained. Samples were taken each day and each sample was inspected visually for colour and clarity. Samples were stored at −20°C prior to their analysis for microbiological assays.

Preparation of PLGA (75:25) (Mw 136 000) microspheres. Teicoplanin incorporated PLGA microspheres were prepared by a modified emulsion/solvent evaporation process by the research group (Mehta et al. 1994, Arica et al. 1999, Hincal and Çalış 2000, Bozdağ et al. 2001, Eroğlu et al. 2001). Blank microspheres were also prepared in a similar way in order to be used as controls in release studies. Microspheres were sterilized by exposing to γ-irradiation in which γ-irradiation (25.4 kGy) was supplied from a ^{60}Co-γ source (Arica et al. 1999, Tunçay et al. 2000, Bozdağ et al. 2001, Eroğlu et al. 2001). The sterility of the products was checked using the USP XXIII procedure.

Preparation of chitosan gel. Chitosan gel which was used during in vivo studies for implantation of microspheres, prepared by dissolving chitosan in 1% lactic acid solution. Afterwards, chitosan gel was sterilized by autoclaving at 121°C for 15 min (Wade and Weller 1994).

Characterization of PLGA microspheres

Particle size distribution. The particle size distribution was measured using a Coulter Multisizer II (Coulter-England). Microspheres were suspended in distilled water containing 0.1% Tween 80. After stirring and ultrasonicating for 10 s, particle size analysis was performed.

Yield. The total microsphere yield was calculated gravimetrically on the basis of polymer/drug recovery.

Surface morphology. Scanning Electron Microscopy (SEM) evaluation of the PLGA microspheres was carried out to examine surface morphology. Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a 150 Å thick layer of gold in a Bio-Rad apparatus. A Scanning Electron Microscope (Jeol-SEM ASID-10 Device in 80 KV) was used to evaluate surface characteristics.
Drug content. Ten milliliters of pH 7.4 phosphate buffer was added to 50 mg of teicoplanin-PLGA microspheres and kept in an ultrasonic bath for 5 min. After the removal of supernatant by centrifugation, the teicoplanin amount was measured by the agar diffusion method to determine the antibiotic present on the surface of the microspheres. Following this, the polymer was dissolved in 5 ml of methylene chloride and to this solution 10 ml of pH 7.4 phosphate buffer was added in which teicoplanin was extracted for 2 h. Finally, after the evaporation of methylene chloride, polymer was precipitated and teicoplanin amount in microspheres was measured. Total content of the drug present on the surface and inside the microspheres were calculated by this procedure.

In vitro release. For in vitro release studies; 30 mg of PLGA microspheres were weighed accurately in separate polypropylene vials for each time point and tubes were placed in a thermostated bath shaken continuously at 40 cpm at 37°C. Samples were taken every 24 h and, after the centrifugation at 6000 rpm, supernatant was removed and drug content was determined by zone inhibition measurements, microbiologically.

In vivo studies

The in vivo study was carried out on three groups of 8 month-old skeletally mature New Zealand white rabbits, weighing ~2.5–3.5 kg (n = 18). The animals were housed in individual cages and were provided with a standard laboratory rabbit diet and water ad libitum. The experimental protocol was reviewed and approved by the Hacettepe University Local Animal Ethics Committee (No: B.30.2.HAC.0.01.00.04).

Before the operation, rabbits were anaesthetized with xylazine HCl (0.1 cc/kg) and ketamine HCl (10 mg/kg). The delivery system was implanted into the femoral condyle of rabbits through an intra-articular defect of 4 × 10 mm (figure 1).

In vivo experimental groups were summarized in table 1. The animals were randomized into three groups. In group I, plain microspheres were implanted into the defect, while in Group II teicoplanin-loaded PLGA microspheres were implanted into the defect and in Group III teicoplanin-loaded PLGA microspheres were incorporated in chitosan gel media. After implantation, regular samples of joint fluid were obtained in 24 h, in days 2, 3, 5 and weekly thereafter. The anti-microbial activity of this fluid was also measured using an agar gel plate technique, as described in the microbiological assay. Fischer’s exact $\chi^2$ test was used for statistical analysis.

Results and discussion

Validation of assay

It is usual approach to demonstrate the activity of the anti-microbial agents by testing their inhibitory effect on micro-organisms under suitable conditions (USP XXIII 1995). Therefore, the activity of teicoplanin has been tested in vitro against various micro-organisms for this purpose. In the literature, MICs determined for various bacterial organisms have been reported (Lewis et al. 1988, Yenice and Çalış 2001). In these studies, Staphylococcus aureus, the most causative micro-organism in osteomyelitis, has been used. The microbiological method has been validated following the literature.
Figure 1. Stages of implantation procedure. (a) Lateral femoral condyle of rabbits was exposed and a standard $4 \times 10$ mm transverse hole was drilled, (b) implantation of teicoplanin loaded microspheres on which chitosan gel is placed, (c) closing of the layers.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ($n = 2$)</td>
<td>Blank PLGA microspheres</td>
</tr>
<tr>
<td>2 ($n = 8$)</td>
<td>Teicoplanin-loaded PLGA microspheres</td>
</tr>
<tr>
<td>3 ($n = 8$)</td>
<td>Teicoplanin-loaded PLGA microspheres incorporated in chitosan gel</td>
</tr>
</tbody>
</table>
After the evaluation of the obtained data, it has been concluded that the microbiological method appeared as a reliable alternative quantitative assay for teicoplanin. Similarly, it has also been demonstrated previously that microbiological assay of certain antibiotics had given comparative results to HPLC or Fluorescence Polarization Immunoassay (FPIA) Awni et al. 1991).

**Stability study**

As a result of the accelerated stability study, it has been presented that, at the end of the 6th month, bioactivity of teicoplanin decreased by 99%, in relation with the reduction of active teicoplanin, an increase was observed in MIC values (figure 2, table 2).

**Microsphere characterization**

Characterization data of microspheres are summarized in table 3. The total yield of microspheres depended on the ability of the process to prevent aggregation

![Figure 2. Log of remaining teicoplanin vs time.](image)

**Table 2. MIC values (µg/mL) vs time in accelerated stability study.**

<table>
<thead>
<tr>
<th>Time (week)</th>
<th>MIC value (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>&lt; 0.12</td>
</tr>
<tr>
<td>1</td>
<td>0.24</td>
</tr>
<tr>
<td>2</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>0.48</td>
</tr>
<tr>
<td>4</td>
<td>0.48</td>
</tr>
<tr>
<td>5</td>
<td>0.97</td>
</tr>
<tr>
<td>6</td>
<td>3.9</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>24</td>
<td>40</td>
</tr>
</tbody>
</table>
of the microspheres and on the solubility of the polymer in the extraction and washing media. Yield value was found as 66%.

**Surface morphology**

Examination of SEM photographs of the microspheres revealed that, the surfaces were spherical and more or less porous (figures 3(a) and (b)). At the end of the release studies, the pores seemed to be enlarged while the number of pores appeared to increase, but the microparticles maintained their spherical shape. Previous observations have demonstrated that the polymer molecular weight and the co-solvent used in the preparation procedure were the factors influencing the porous structure of the particles. High molecular weight polymers were shown to yield less porous microparticles, thus PLGA 136 000 has been used in this study for obtaining microspheres of appropriate surface characteristics (Hincal and Çalış 2000, Tuncay et al. 2000). Another influencing parameter for a porous characteristic is the nature of the co-solvent. Methanol has been demonstrated to give porous microparticles (Mehta et al. 1994, Hincal and Çalış 2000, Tuncay et al. 2000). However, due to the solubility problem of teicoplanin in methanol, DMSO was used during the manufacturing process of the microparticles. Because of its higher evaporation temperature, DMSO was believed to produce a less porous particle surface. It was also assumed that a less porous microparticle would achieve a controlled release property (Hincal and Çalış 2000).

**Particle size**

Particle size distribution of the teicoplanin loaded PLGA 75:25 microparticles was found to be ~29μm. No particle size limitations were encountered for bone implant preparations. Nevertheless, large particle size preparations could be advantageous in terms of easier application during surgical operation.

### Table 3. Characterization of teicoplanin-loaded PLGA 75:25 microspheres.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Yield value (%)</th>
<th>Encapsulation efficiency (%)</th>
<th>Particle size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA microspheres</td>
<td>66.00</td>
<td>1.73</td>
<td>29 ± 4.57</td>
</tr>
</tbody>
</table>

![Figure 3. SEM photographs of the teicoplanin loaded (a) before release, (b) after release.](image_url)
Drug content

A theoretical drug loading of 10% w/w was readily achievable using teicoplanin, with an encapsulation efficiency of 1.7%. In the literature, problems related to the efficient incorporation of water soluble active substances into biodegradable polymer matrices using simple o/w emulsification with solvent evaporation was originated to a great extent from the separation and/or removal of water soluble material into the aqueous continuous phase (Arshady 1990, Hermann and Bodmeier 1998). These results were independent of the drug solubility and reflected the microsphere preparation technique (Hincal and Çağlar 2000).

In vitro release

It has been observed that release studies for formulation loaded with heat sensitive drugs such as antibiotics or peptide/proteins were carried out by replacing small quantities of (1–1.5 mL) release medium in microtubes for each sampling time (Lin et al. 1999). Since 4–6 weeks of release time has been targeted and due to the labile structure of the antibiotic used in this study, it has been obligatory to use this modified release method. As predicted, PLGA 75:25 microspheres achieved a 5-week release time (figure 4). The initial burst effect observed by the immediate release of 40% drug was related to the teicoplanin adsorbed to the surface of the microspheres. High release rates at the beginning of an antibiotic therapy were determined to be very important, since the efficiency of antibiotics often depended on high drug concentrations at the beginning of a therapy at the site of infection (Schmidt et al. 1995). It was observed (figure 4) that, at the end of the 7th day, 65% of drug was released while, after 2 weeks, the amount released reached up to 80%. Teicoplanin release was shown to be terminated at the end of the 5th week. The relationship between released drug from PLGA microspheres and remaining drug in PLGA microspheres was shown in figures 4 and 5. The amount of residual drug decreased with an increasing amount of drug released as expected.

Figure 4. In vitro release profile of teicoplanin-loaded PLGA microspheres.
In vivo studies

This method provided a prompt release of therapeutically active antibiotic to the synovial fluid and the entrapment within the bone was prevented. Two formulations prepared (teicoplanin-loaded microspheres and teicoplanin-loaded microspheres dispersed in chitosan gel) released antibiotic to the medium for 10 days (figure 6). A statistical difference between the above mentioned two groups was evaluated by Fisher’s exact $\chi^2$ test and difference was found to be statistically insignificant ($p > 0.05$). Samples from the synovial fluid of the rabbits could not
be removed for the 2nd and 3rd weeks of the experiments due to infection at the implantation site.

*In vivo* analysis revealed that the drug concentration exceeded the MIC for the test organism throughout 2 weeks without inducing serum toxic levels. Antimicrobial activity was not detected in the group I samples which corresponded to the blank microspheres (table 1). At the end of the 2nd week of the *in vivo* studies, 86% of animals had wound infection complications. It was reported in the literature that, following the administration of synthetic polymers, an increase in degradation products has led to synovitis and sterile abscess formation in 5–20% of cases (Athanasiou *et al.* 1998, Cornell 1999). This situation and repeated needle punctions might be logical approaches for explaining the high rate of infections observed in animals. Another interesting observation was the recovery in 92% of animals without any treatment. Furthermore, anti-microbial activity could still be detected in ~ 40% of animals. So, one had the experience that the microbiological analysis of the antibiotic teicoplanin from the synovial fluid did not seem to be practical, but troublesome for *in vivo* testing. This has appeared as a major drawback in the *in vivo* study.

**Conclusion**

In conclusion, *in vitro* data obtained from teicoplanin-loaded PLGA (75:25) microspheres was quite satisfactory, since the duration of *in vitro* release was 5 weeks, as it was planned. Furthermore, it was also noteworthy that the microbiological assay method which was analytically validated could be used throughout the *in vitro* studies for quantitative determination of the antibiotic. Although the *in vivo* data seem to be promising, it was experienced that quantitative determination of the antibiotic from the synovial fluid was troublesome and also *in vivo* techniques should be chosen by caution to prevent potential handicaps.

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**References**


Teicoplanin-loaded biodegradable microparticles


