

Antibiotic Microspheres: Preliminary Testing for Potential Treatment of Osteomyelitis

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Osteomyelitis is a difficult problem for orthopaedic surgeons. The current standard of treatment requires high doses of antibiotic to be administered parenterally, which can damage vital organs. A local drug delivery system, which targets only the infected tissues, would eliminate some of the complications associated with extended courses of parenteral antibiotic treatment. In the current study, biodegradable microspheres were manufactured from a high molecular weight copolymer of 50% lactic and 50% glycolic acid and the antibiotic tobramycin. Various formulations of microspheres were tested for in vitro elution characteristics to determine the optimum formulation for linear release of antibiotic for at least 4 weeks. The optimal formulation then was implanted into a pouch created in the quadriceps muscle of mice to evaluate the in vivo elution of the antibiotic and the inflammatory response elicited by the microspheres. Results indicate that a sustained linear release of antibiotic from the microspheres is possible for a period of at least 4 weeks and that the inflammatory response was within levels required for the microspheres to be considered biocompatible.

Osteomyelitis has long been a difficult problem for orthopaedic surgeons to treat. Infection rates after orthopaedic surgery range from 0.4% to 6%, depending on the type of surgery that is done.^{2,7,9,24} Currently, the standard treatment of osteomyelitis includes debridement of infected tissues, dead space management, and 4 to 6 weeks of parenteral antibiotics. Numerous antibiotics have been reported to be useful in the treatment of osteomyelitis, and these antibiotics vary in their ability to reach infected bone tissues.¹⁸ In a study of experimental *Staphylococcus aureus* osteomyelitis, the concentration of antibiotic in the infected bone ($\mu\text{g/g}$) relative to the serum concentration ($\mu\text{g/mL}$) ranged from 98.3% for clindamycin to 3.7% for cephalothin.¹⁸ In the same study, the concentration of tobramycin in infected bone tissues was only 9.1% of the serum concentration. Therefore, to penetrate the infected bony tissues with an adequate dose of antibiotic, large doses have to be used, which can result in nephrotoxicity, ototoxicity, or gastrointestinal complications.

A local drug delivery system, which targets only the infected tissues, would eliminate some of the complications associated with extended courses of parenteral antibiotic treatment. In 1970, Buchholz and Engelbrecht⁵ advocated the use of antibiotic-containing bone cement for the treatment of osteomyelitis, and this treatment has been studied extensively and used since that time. Polymethylmethacrylate (PMMA) containing antibiotic has been shown to be effective for prophylaxis²⁰ and the treatment of osteomyelitis.^{11,23}

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Received: June 11, 2002.

Revised: October 21, 2002; December 16, 2002.

Accepted: January 10, 2002.

DOI: 10.1097/01.blo.0000093920.26658.ae

despite the fact that the antibiotic is released from the cement nonlinearly during a diminished period.¹⁵ In addition, a second surgery may be required to remove the cement because it is not biodegradable and may become a nidus for future infections. There is some evidence that bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis* form a biofilm that limits the activity of antibiotics.⁴ This biofilm can adhere to PMMA, which may make it extremely unsuitable as a drug delivery vehicle.⁴

Many types of biodegradable drug delivery systems have been proposed for antibiotics including calcium sulfate, polymers of lactic or glycolic acid, and collagen.^{1,6,12,14-17,19,21-23,26,27} Implantable drug delivery carriers must deliver the antibiotic at a steady rate for at least 6 weeks, remain immobilized at the site of implantation, and be biocompatible. To remain at the infection site, the particles must be large enough to not be phagocytosed by macrophages. Green et al¹⁰ determined that 0.3- to 10- μm polyethylene (PE) particles elicited the most response from macrophages.

The results of two experiments using microspheres created using a water-in-oil-in-water double-emulsion-solvent-extraction technique are presented. The microspheres are made of varying amounts of poly (DL-lactic-co-glycolic acid) (PLGA), poly (ethylene glycol) (PEG) and tobramycin. In the first experiment, the *in vitro* elution characteristics of various formulations of microspheres were studied. In the second experiment, the *in vivo* elution was studied using a mouse muscle pouch model.

MATERIALS AND METHODS

The PLGA used was a high molecular weight copolymer of 50% lactic and 50% glycolic acid (Medisorb[®], Alkermes, Cincinnati, OH) with a reported polymer weight average molecular weight of 79,000. Polyethylene glycol with nominal molecular weight of 4600 and polyvinyl alcohol with a molecular weight of 25,000 were purchased from Sigma Aldrich (St Louis, MO).

Tobramycin (Nebcin[®], Eli Lilly, Indianapolis, IN) was purchased in powder form, and all remaining chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Microsphere Preparation

Microspheres were prepared in many ratios of PLGA to PEG to tobramycin using a double emulsion-solvent extraction technique described previously.⁸ The size distribution of the microspheres was measured with a Coulter counter multisizer (model 0646, Coulter Electronics, Hialeah, FL) after suspending the particles in an Isoton II solution (Coulter Electronics).

The entrapment efficiency was defined as the weight of antibiotic actually entrapped within the microspheres divided by the weight used in the preparation of the microspheres. The entrapment efficiency of each formulation was determined in duplicate using an established solvent extraction technique.⁸ Ten milligrams of microspheres were dissolved in 1 mL dichloromethane for 6 hours at room temperature. The tobramycin then was extracted from the organic phase to the aqueous phase by mixing 1 mL phosphate buffered saline (PBS) and removing the aqueous portion. This was repeated every 6 hours for 24 hours and all aqueous aliquots were tested for tobramycin concentration. The sum of the weights of tobramycin found in each aliquot was presumed to be the total entrapped amount of antibiotic.

All tobramycin concentrations were determined using fluorescence polarization immunoassay (Abbott TDx System, Abbott Laboratories, Abbott Park, IL). Sensitivity of the tobramycin assay is defined as the lowest measurable concentration, which can be distinguished from 0 with 95% confidence, and was determined to be 0.18 $\mu\text{g}/\text{mL}$.

In Vitro Elution Rate Determination

By dry weight, the percentage of PEG in the formulations was either 0% or 5%, and the percentage of tobramycin was either 1%, 5%, or 10%. Six different formulations were studied for tobramycin elution rates. Twenty-five milligrams

of microspheres were measured and placed into 2 mL glass vials containing 1 mL PBS. Each microsphere formulation was tested in triplicate and placed in a water bath at 37° C. After 24 hours, the vials were centrifuged and the supernatant was removed for tobramycin assay. One milliliter of PBS was added to the vials and the vials were replaced in the water bath. This was repeated once daily for 1 week and then every second day for 3 additional weeks.

In Vivo Drug Release Characteristics

Microspheres with 10% tobramycin and either 0% or 5% PEG were studied in a mouse muscle pouch model. Sixty adult female Institute of Cancer Research (ICR) mice, weighing 20 to 24 g, were used for this investigation. Each animal was anesthetized using ketamine (150 mg/kg) and xylazine (6 mg/kg) intraperitoneal injection. A small incision was made over the right quadriceps muscle and a small pouch was made in the muscle by blunt dissection. In 30 mice, 5 mg of microspheres containing 10% tobramycin and 0% PEG were implanted into the pouch; in the remaining 30 mice, microspheres containing 10% tobramycin and 5% PEG were implanted. Each pouch was closed with a nonabsorbable suture to mark the location. The skin was closed with a resorbable suture. All animals ambulated normally throughout the study, and no signs of local inflammation (swelling, tenderness) were visible. All animal procedures were approved by the Animal Welfare Committee at the authors' institution.

For each of the two microsphere formulations tested, the mice were divided into five groups of six mice each and sacrificed sequentially at 1 day, 4 days, 7 days, 22 days, and either 33 or 40 days after surgery. At sacrifice, the scarred incision was reopened and the pouch was located by the suture. Approximately 0.1 g of tissue surrounding the suture was removed. Half of the tissue was placed in formalin for subsequent histologic evaluation.

The remaining half of the tissue was weighed and placed in 0.5 mL PBS and macerated. The tissues from three mice in each group were

pooled randomly together in each vial such that there were two vials for each point per group. The tissue was incubated for 2 hours at 37° C. After incubation, the vial was centrifuged and the supernatant was filtered for tobramycin analysis. Tobramycin concentration is presented as weight of tobramycin per weight of muscle tissue. Because the tissue contained some microspheres, the amount of tobramycin found could have been from either tobramycin in the muscle tissue at the time of sacrifice or from tobramycin released from the microspheres during tissue incubation.

The preserved tissue was cut into 5- μ m sections and stained with hematoxylin and eosin. Each slide was graded for inflammation by a pathologist who had no knowledge of the study group distribution. The pathologist graded each slide according to the following scale: 1 for no or minimal inflammation, 2 for moderate inflammation, and 3 for marked or severe inflammation.

RESULTS

In Vitro Results

The in vitro elution of the six microsphere formulations is shown in Figure 1. In this figure, the cumulative amount of drug released was divided by the total amount present in the implanted microspheres. Therefore, when an elution curve reached 0.8 on the y-axis, 80% of the antibiotic present in the microspheres had been released. The entrapment efficiency for each formulation of microsphere ranged from 40.24% to 61.8% as shown in Table 1. In general, adding PEG increased the entrapment efficiency. All microspheres were $20 \pm 1.6 \mu\text{m}$ (mean \pm standard deviation) in diameter on average.

Each formulation had a large initial release of tobramycin in the first 24 hours, followed by a few days of lowered release and then a few weeks of nearly steady release. Linear fits of the elution curves during the 7- to 28-day period showed correlations ranging from $r^2 = 0.7748$ to 0.9770 , indicating that the release of antibiotic is linear during this period. Table 1 shows the calculated average linear release of tobramycin for each

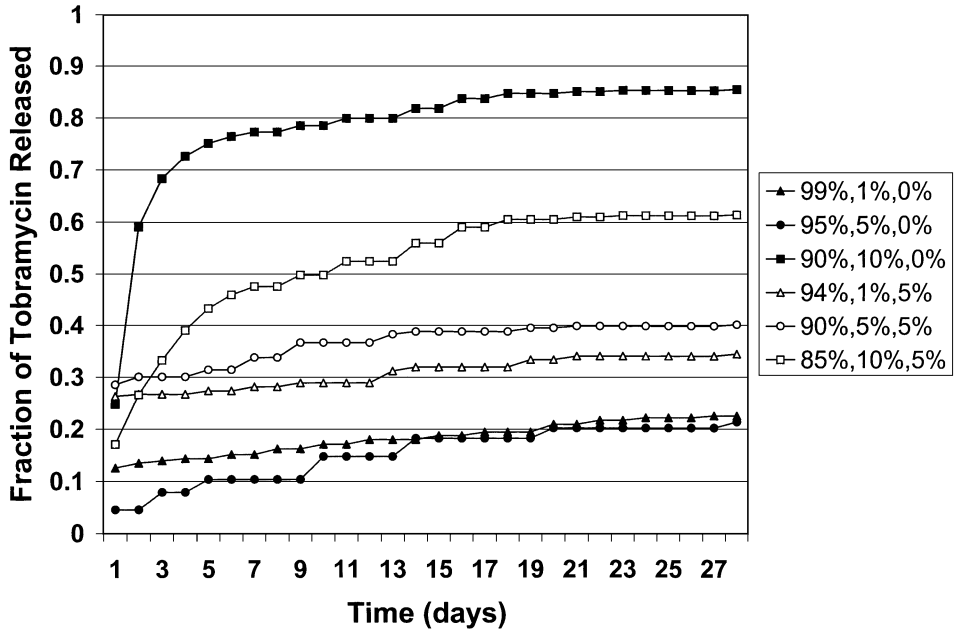


Fig 1. In vitro releases of tobramycin from different microsphere formulations are shown as a function of time. Microsphere formulations are given as three percentages: percent of PLGA, percent of tobramycin, and percent of PEG, by weight. The release is normalized to the total amount of tobramycin entrapped in the microspheres.

formulation for Days 7 through 28 in absolute amounts and percentage of total amount of drug.

A repeatability study was done in which two of the formulations were manufactured more than 1 year apart. The in vitro elution rates for these experiments are shown in Figure 2. Again, the cumulative weight of tobramycin released was divided by the total amount present to give a percentage shown on the y-axis.

In Vivo Results

The in vivo tobramycin concentrations are shown in Figure 3 for the two formulations tested. In this figure, the amount of tobramycin was divided by the weight of the tissue sample. The minimum inhibitory concentration of tobramycin against *Staphylococcus aureus* is shown for comparison. The histologic scores for the quadriceps tissue for one formulation are shown in Table 2.

TABLE 1. Microsphere Characteristics and In Vitro Elution

Microsphere Formulation				Average Release	
PLGA (percent)	Tobramycin (percent)	PEG (percent)	Entrapment Efficiency	µg/day	percent/day
99	1	0	42.8%	0.3852	0.48
95	5	0	N/A	2.3586	0.37
90	10	0	45.8%	4.4510	0.41
94	1	5	61.8%	0.5131	0.33
90	5	5	40.2%	1.3415	0.27
85	10	5	52.4%	8.7916	0.67

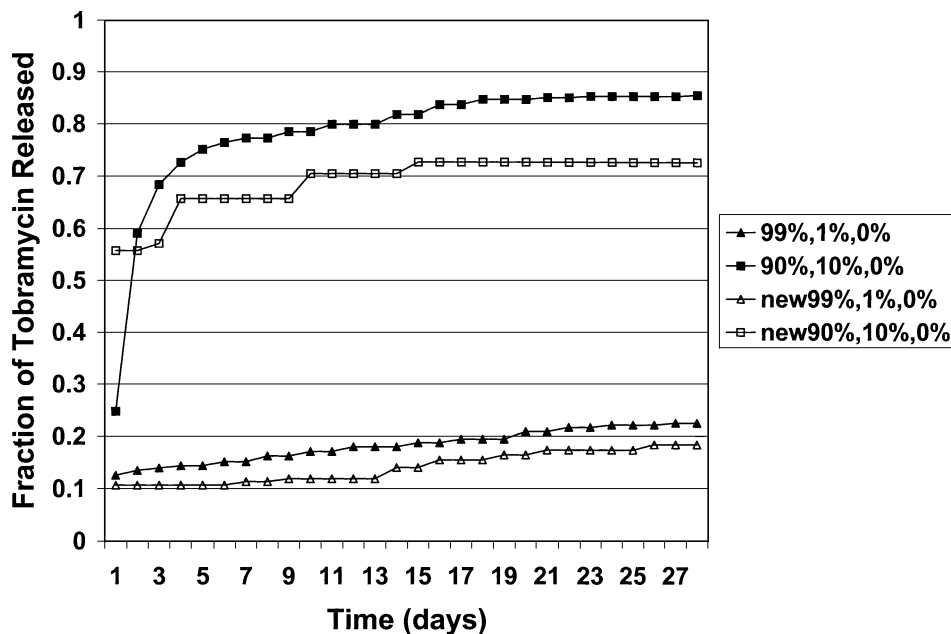


Fig 2. Each formulation of microspheres was produced twice, more than 1 year apart. The curves show the reproducibility of the manufacturing method. Microsphere formulations are given as three percentages: percent of PLGA, percent of tobramycin, and percent of PEG, by weight. The word new indicates the second batch manufactured at least 1 year after the first.

DISCUSSION

The results of the *in vitro* studies show that changing the antibiotic concentration and the concentration of PEG can alter the elution characteristics of the antibiotic. In general, increasing the concentration of either component decreased the rate at which the antibiotic was released, although the initial burst of drug released increased with increasing antibiotic or PEG concentration. In all formulations, the release rate leveled off to a near linear rate after the first week and remained steady for the next 3 weeks. At these linear release rates, it was determined that the formulation with 90% PLGA-10% tobramycin-0% PEG would have released all of the antibiotic in 60 days. By contrast, the formulation with 99% PLGA-1% tobramycin-0% PEG would take approximately 186 days to release all of the antibiotic. As shown in Figure 2, the different microsphere formulations can be reproducibly manufactured.

Results of the *in vivo* study show that these microspheres do not elicit an extreme inflammation response. The inflammation did increase to the highest level by Day 3, but returned to minimal levels by Day 21 and remained there for the subsequent times. This short-term inflammatory response is associated with the implantation of any biomaterial but the biomaterial can be considered biocompatible if the response recedes within 21 days.^{3,25} This inflammation was localized to the implant site and did not produce macroscopic signs of inflammation nor did it affect the animals' appetite or ambulation.

The *in vivo* data showed higher tissue concentrations of tobramycin for the 90% PLGA-10% tobramycin-0% PEG formulation. Although the tissue levels were measurable for the 85% PLGA-10% tobramycin-5% PEG formulation throughout the study, they remained at or below the minimum inhibitory concentration for *Staphylococcus aureus* in the second through fourth weeks. By contrast, the 90% PLGA-10% tobramycin-0% PEG

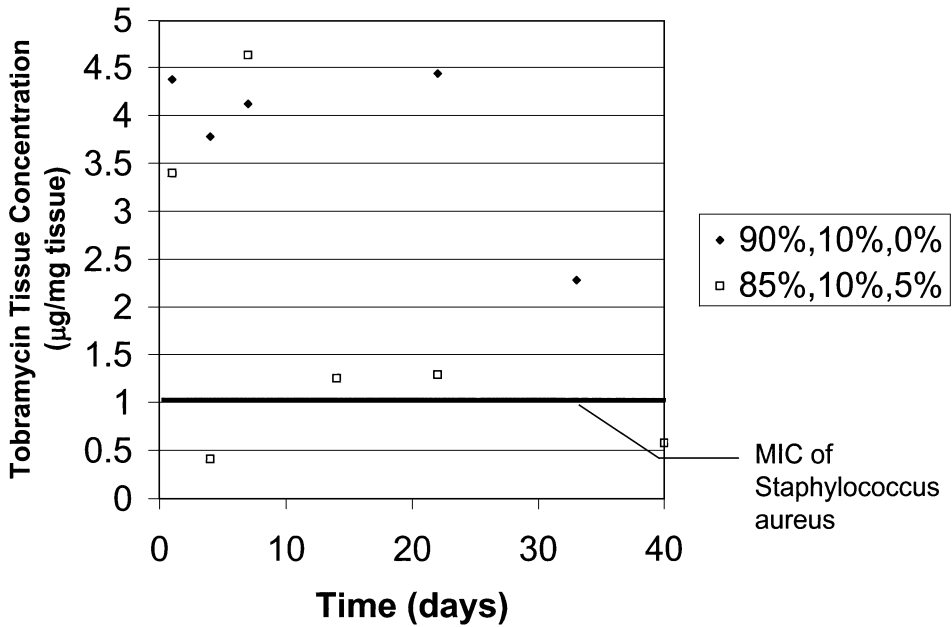


Fig 3. Tobramycin is given as a concentration of weight of tobramycin per weight of muscle tissue. For comparison, the minimum inhibitory concentration (MIC) of tobramycin against *Staphylococcus aureus* is shown on the graph.

formulation resulted in tissue concentrations at least twice the minimum inhibitory concentration for the entire study period. This was an unexpected result because the *in vitro* elution characteristics showed a larger linear release rate of tobramycin for the 85% PLGA-10% tobramycin-5% PEG formulation during Days 7 though 28, and it was presumed that this would translate to higher

tissue levels. One obvious and simplistic explanation is that the microspheres degraded differently *in vivo* than *in vitro* and the release rates were altered. An alternate explanation is that the higher initial release of antibiotic during the first week for the 90% PLGA-10% tobramycin-0% PEG formulation contributed to the higher tissue levels found *in vivo* throughout the study period.

TABLE 2. Histologic Scoring for Quadriceps Tissue

Time (Days)	Histologic Inflammation Score 90% PLGA-10% tobramycin-0% PEG
1	1
4	3
7	3
21	1
30-40	1

Grading Scale: 1, no or minimal inflammation; 2, moderate inflammation; 3, marked or severe inflammation

Microspheres were visible with the histologic examination indicating that the microspheres remain at the site of implantation for at least 30 days, and measurable tobramycin levels were found in the tissue for both formulations of microspheres throughout the length of the study. This indicates that the microspheres are too large to be phagocytosed and remain at the site of implantation. Although macrophage response was not specifically examined in this study, Horisawa et al¹³ determined that microspheres made of PLGA in the same size range as those produced in the current study were not phagocytosed.

The results of the current study suggest that microspheres made of PLGA and tobramycin,

with or without PEG, make a suitable biodegradable drug delivery system. These microspheres do not elicit an undesirable inflammatory response, and the formulation can be adjusted to vary the release kinetics of the antibiotic. The microspheres deliver the antibiotic at a near-linear rate for at least 4 to 6 weeks. Some methods of local antibiotic delivery such as cement beads can become a physical barrier to tissue growth and regeneration into debrided defects. The microspheres presented here remain at the site of implantation but are small and biodegradable and therefore do not act as a barrier in cases where tissue regeneration is desired.

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